

Identification of a Potential Effector Function for IgE Autoantibodies in the Organ-Specific Autoimmune Disease Bullous Pemphigoid

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Bullous pemphigoid (BP) is an autoimmune skin disease characterized by autoantibodies against the hemidesmosomal protein BP180. In addition to IgG autoantibodies, IgE class autoantibodies have been reported in BP patients. Because animal models utilizing only IgG antibodies do not totally replicate human BP, we examined the specificity and potential relevance of IgE autoantibodies in this disease. Thirty BP patients participated in these studies. Serum IgE was measured and the IgE specificity was determined by immunoblotting. Double labeling Immunofluorescence was performed using combinations of specific antibodies to human mast cell tryptase, IgE and BP180. BP180-stimulated histamine release was measured from basophils of untreated BP patients (n = 9), BP patients undergoing immunosuppressive therapy (n = 9) and controls (n = 16). Elevated IgE levels were found in 70% of untreated BP patients. IgE autoantibodies directed against

BP180 were detected in 86% of untreated patients and in all but one of these patients the IgE reacted with the NC16A domain of BP180. IgE-coated mast cells were detected in perilesional skin of the BP patients. Moreover, BP180 peptides were detected on these mast cells. BP180-stimulated histamine release was significantly higher in basophils obtained from untreated BP patients compared with control basophils (p = 0.006) or from treated BP patients (p = 0.01). These findings support the hypothesis that IgE autoantibodies are involved in the pathogenesis of BP. IgE and IgG BP autoantibodies share the same antigenic specificity. Antigen-specific degranulation of basophils and/or mast cells from BP patients suggests a mechanism by which IgE may contribute to lesion development. **Key words:** Bullous pemphigoid/IgE/autoimmunity/collagen XVII. *J Invest Dermatol* 120:784–788, 2003

Bullous pemphigoid (BP) is an autoimmune disease characterized by subepidermal blistering. The first evidence of the immunologic basis of this disease was obtained in 1967 by Jordon *et al* (1967). Immunoglobulins and C3 were found to be deposited along the basement membrane zone (BMZ) in the skin of BP patients. Anti-BMZ antibodies of the IgG class were also found in the sera of these patients.

BP180, a transmembrane member of the collagen protein family (also designated type XVII collagen) has been shown to be the primary antigenic target of BP autoantibodies (Diaz *et al*, 1990). BP180 is expressed on the basal surface of basal epidermal keratinocytes and is a constituent of the hemidesmosomal complex, a cellular structure that functions in dermal–epidermal adhesion (Giudice *et al*, 1993). The major disease-associated epitopes

of BP180 have been mapped to the NC16A domain, a membrane-proximal noncollagenous stretch of its ectodomain (Zillikens *et al*, 1997).

Animal model studies have provided strong evidence that IgG antibodies reactive with the NC16A domain of BP180 play a direct role in the onset and progression of disease. When antibodies generated against the murine ortholog of BP180 are injected into neonatal BALB/C mice, the mice develop a skin disease with many of the key immunopathologic features of BP (Liu *et al*, 1993). The development of lesions is dependent upon activation of complement, recruitment of neutrophils, and release of proteases, including neutrophil elastase and gelatinase B, at the lesion site (Liu *et al*, 1995a, b, 1997, 1998, 2000). Two key features of the human disease, however, are not reproduced in the BP mouse model: eosinophilic infiltration of the dermis and spontaneous blistering. In human BP, eosinophils are a regular histologic feature (Dvorak *et al*, 1982; Nishioka *et al*, 1984), whereas in the mouse model the infiltrate is composed almost exclusively of neutrophils. In the passive transfer mouse model, frictional pressure is applied to the injection site in order to see detachment of the skin grossly. In contrast, mechanical pressure is not recognized as a strong contributor to blister development in BP. These findings led us to investigate whether autoantibodies of the IgE class play a part in the development of the complete clinical picture of BP.

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Whereas most work on BP has focused on IgG autoantibodies, several previous studies have identified an IgE response in this disease as well. IgE was detected at the cutaneous BMZ in 25% of BP patients (Provost and Tomasi, 1974) and elevated circulating IgE levels (with unknown specificities) were observed in 70% of BP patients (Arbesman *et al*, 1974; Bowszyc-Dmochowska *et al*, 2000). More recently, circulating IgE class antibodies against BP180 were found in the majority of untreated BP patients (Delaporte *et al*, 1996; Christophoridis *et al*, 2000; Dopp *et al*, 2000).

This study was designed to characterize further the pathologic relevance of IgE antibodies in BP. We systematically evaluated 32 BP patients for the presence and specificity of IgE class autoantibodies. We also examined the skin of these patients for the presence of dermal mast cells coated with anti-BP180 surface IgE. Finally, circulating basophils obtained from these patients were analyzed for the ability to degranulate in response to recombinant BP180. Our data show that IgE class antibodies are present in a large number of BP patients and that these autoantibodies may play a part in the pathogenesis of the disease.

MATERIALS AND METHODS

Development of a panel of well characterized BP sera Thirty-two patients with well-characterized BP took part in these studies, 23 of whom had not received any immunosuppressive therapy prior to their immunologic assessment. All patients had clinical and histologic features characteristic of BP. In addition, all patients had direct immunofluorescence (IF) demonstrating IgG and/or C3 at the BMZ and 29 of 32 (91%) had initial positive indirect IF that showed IgG binding to the roof of NaCl split skin (Gammon *et al*, 1984). IgE levels were measured in the serum of the patients by a human IgE capture enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories, Inc. Montgomery, TX).

IF testing Circulating anti-BMZ autoantibodies were detected by indirect IF using salt-split normal human skin as a substrate and serial dilutions of the patient's serum as a primary antibody. Fluorescein-conjugated anti-human IgG or IgE was used as a secondary antibody. For direct IF, 4 μ m cryosections of perilesional BP skin (or controls) were incubated with the fluorescein isothiocyanate (FITC) conjugated anti-human IgG (γ -chain specific) or IgE (ϵ -chain specific) for 30 min at room temperature. After washing, the slides were viewed with a Nikon photomicroscope equipped for epifluorescence.

Skin sections from BP patient biopsies ($n = 5$) were double labeled with Texas red conjugated anti-human mast cell tryptase and either FITC-anti-human IgE (ϵ chain specific), FITC-anti-human IgG (γ chain specific) or HD18, a monoclonal anti-human BP180 antibody (Polha-Gubo *et al*, 1995). As an additional control, double labeling experiments were performed with an additional monoclonal antibody of the same subclass against another component of the BMZ, the α -3 chain of laminin 5. The sections were examined with both FITC (GFP) and Texas Red (DM580) filters and images were taken with a SPOT RT camera. The two images were superimposed using SPOT RT software in order to identify cells labeled with both conjugates.

Immunoblotting of recombinant proteins and skin extracts The recombinant NC16A domain of BP180 was prepared as previously described (Zillikens *et al*, 1997). Affinity purified recombinant NC16A peptide or human keratinocyte extracts were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and immunoblotting was carried out using a standard protocol (Zillikens *et al*, 1997).

Histamine release by BP patient basophils (Botulinska *et al*, 1999) Histamine release from peripheral blood basophils in response to the NC16A domain of BP180 was measured using a commercially available competitive immunoassay (Immunotech, Fullerton, CA). Peripheral blood was drawn from 34 subjects: 16 normal volunteers, nine untreated BP patients with active disease, and nine BP patients who were treated and had minimal or no disease activity. Controls were 16 age-matched patients with other skin diseases. These included seven patients with autoimmune diseases other than pemphigoid: pemphigus vulgaris ($n = 4$), pemphigus foliaceus ($n = 2$), and lupus erythematosus ($n = 1$). Other controls had a variety of other inflammatory or neoplastic skin disorders. Because no differences were seen in any measurement between

these two subsets of the control group they were combined for analysis. 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 (nonimmunologic release), buffer only (spontaneous histamine release), or lyzed 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 pNPP. 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. One control sample was eliminated from analysis because of spontaneous release in excess of 5%.

Statistical analysis Serum IgE levels were compared by the Wilcoxon rank sum test between the untreated BP patients, treated BP patients and controls. The ratio of histamine release by NC16A to the background histamine release was compared among the untreated BP patients, treated patients and controls using the Wilcoxon rank sum test. The Mann-Whitney median confidence interval was used to give a nonparametric estimate of the relative size of the histamine ratios for the three groups. All calculations were performed with SAS statistical software (The SAS Institute, Cary, NC).

RESULTS

BP patients have elevated serum IgE Levels The results of the serum IgE measurements are shown in **Fig 1**. The serum IgE level was elevated in 16 of 23 untreated patients (70%). The mean IgE level of the untreated patients was 399 IU (range 37–1766) with a normal range for this assay of 0–120 IU per ml. The controls from our outpatient clinic ($n = 17$) had a mean IgE level of 31 IU and no control patient had an IgE level above 120 IU. This was a statistically significant difference between controls and untreated patients ($p < 0.0001$). Only three of the nine partially treated patients had IgE levels > 120 IU. Whereas the mean IgE level of the treated patients still showed some elevation with a mean serum IgE of 213 IU, this was only marginally significant when compared with the controls ($p = 0.0125$).

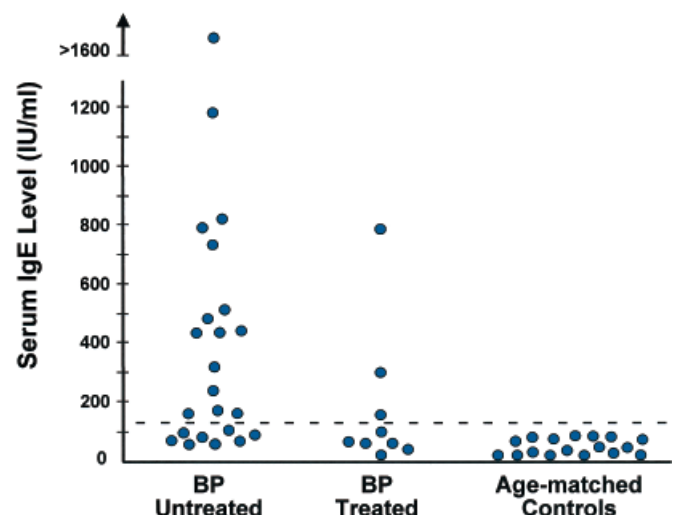


Figure 1. Serum IgE levels in untreated BP patients ($n = 23$), treated patients ($n = 9$), and controls ($n = 17$). Serum IgE levels in untreated BP patients were significantly above the control group ($p < 0.0001$). Three of the BP patients who were treated still had levels above the established upper limit of normal in this assay, 120 IU per ml. The mean IgE level of the control patients was 31 IU per ml and none of the controls had an IgE level above the upper limit of normal for the assay.

IgE reactivity with the NC16A domain of BP180 is present in BP sera The sera of 30 BP patients were tested by immunoblotting for IgE reactivity against the NC16A domain of BP180. Of the 23 untreated BP patients, 16 (70%) had detectable IgE antibodies that reacted with BP180 (Fig 2). The reactivity was with NC16A in 15 of 16 of these patients. One untreated patient whose IgE did not bind NC16A reacted with BP180 protein extracted from human keratinocytes, suggesting this patient reacted with sites on BP180 other than NC16A. In addition, four of seven of the treated patients also had IgE class antibodies that bound to NC16A (57%). These four all reacted with NC16A. Sixteen controls (detailed under histamine release) did not react with NC16A.

BP patients have circulating IgE that binds the BMZ but *in-vivo* IgE is bound to dermal cells Direct IF testing for IgE was performed on perilesional skin of 23 patients in whom biopsy specimens were available and indirect IF on all 32 sera. Whereas no IgE BMZ staining was identified by direct IF, 15 of 18 untreated patients' biopsies showed cells in the papillary and reticular dermis that stained strongly with FITC anti-IgE (Fig 3A) and two of five patients who had received some immunosuppressive treatment prior to biopsy also had cellular staining. In contrast, staining of 102 control samples (skin biopsies submitted to the Clinical Immunodermatology Laboratory with negative routine IF) showed only eight with any detectable cellular staining with anti-IgE. Indirect IF identified seven of 23 untreated sera that contained circulating IgE class antibodies that bound to the BMZ. No treated patient had detectable circulating anti-BMZ IgE.

Mast cells in the dermis of BP patients are opsonized with IgE and have surface-bound BP180 To verify that the cells labeled with anti-IgE in the dermis of BP patients were mast cells, cryosections from five biopsies that had shown cellular staining in the dermis were double labeled with anti-human IgE and anti-human mast cell tryptase. All five specimens showed double labeling of the cells with IgE and tryptase, indicating they were mast cells (not shown). Having demonstrated that IgE-coated mast cells are a common feature in the skin of BP

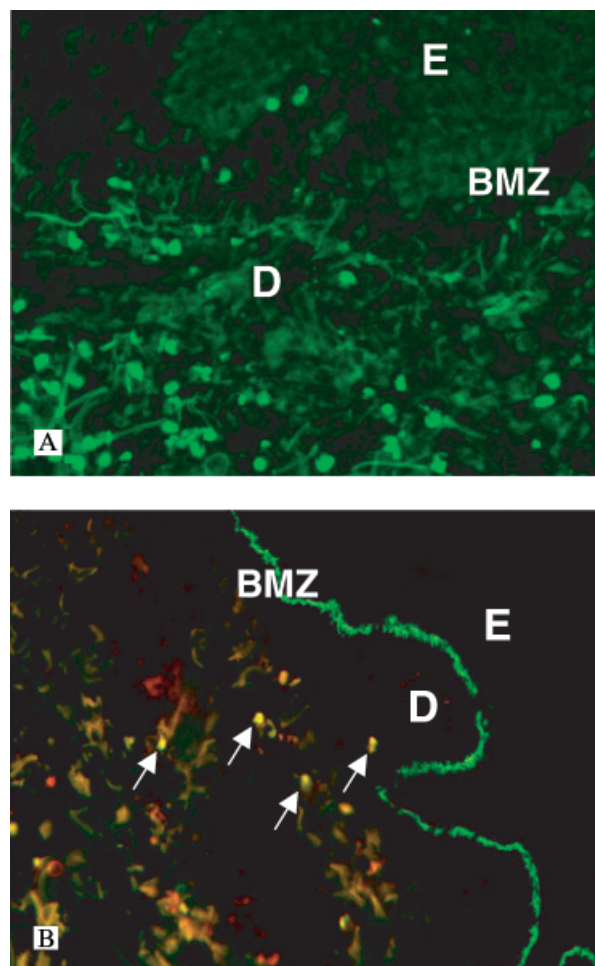


Figure 3. Direct IF of perilesional BP skin. (A) Skin biopsy from a BP patient stained with anti-human IgE. No reactivity is visible at the BMZ, however, numerous cells in the dermis are stained. (B) Double immunofluorescent labeling of the same specimen with anti-human mast cell tryptase and a monoclonal antibody against BP180, HD-18. The BMZ stains green with HD-18 due to the presence of BP180 that is normally present. The yellow cells in the dermis (arrows) are labeled with both anti-human mast cell tryptase and HD-18. This indicates that these mast cells have fragments of BP180 on their surface.

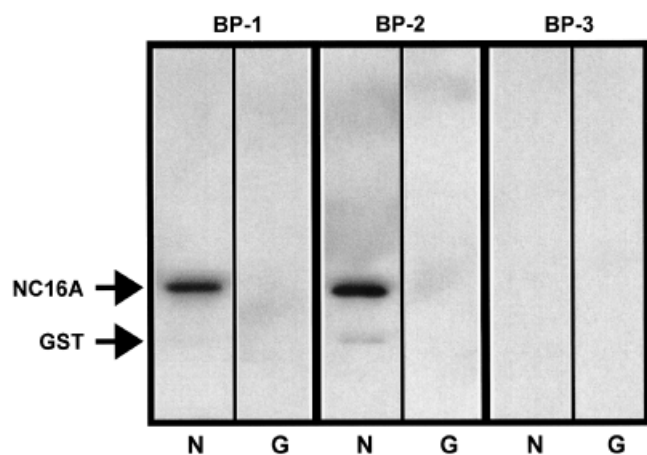


Figure 2. BP IgE reactivity with BP180 NC16A fusion protein. Sera of 30 BP patients were tested for IgE reactivity against the NC16A domain of BP180 by immunoblotting. This representative blot shows the reactivity of sera from three BP patients. The sera were tested against NC16A (lanes marked N) and GST (lanes marked G). Arrows on the left indicate the expected location of reactivity with NC16A or GST. BP patients An-4 and Jo-8 both demonstrate strong IgE reactivity with NC16A and none with GST. The third patient, Ho-12, showed no reactivity with either NC16A or GST. Sixteen of 23 untreated BP patients had detectable IgE antibodies that reacted with NC16A, and four of seven treated patients also had IgE antibodies against NC16A.

patients, we looked for the presence of antigen on the surface of these cells. The same five biopsies were double labeled with anti-human mast cell tryptase and HD-18, a monoclonal antibody that recognizes BP180 (Polha-Gubo *et al*, 1995). Cells that labeled with both the antibodies to tryptase and HD-18 were found in five of five BP biopsies (Fig 3B). This suggests that mast cells in the dermis of BP patients have BP180-specific IgE on their surface and that antigen is also present on the surface of the cells. The control monoclonal antibodies against the $\alpha 3$ chain of laminin 5 labeled the BMZ of the biopsies. No dermal cells labeled with the anti-laminin 5 antibody and no double labeling with anti-human tryptase and anti-laminin 5 was seen in any of these biopsies (data not shown).

In order to ensure that the binding of BP180 on the surface of the mast cells was due to IgE rather than IgG (as under rare circumstances mast cells can express the IgG receptor, Fc γ RI (Okayama *et al*, 2000), we double labeled skin biopsies from BP patients ($n = 5$) with FITC conjugated anti-IgG (γ -chain specific) and anti-human mast cell tryptase. No double-labeled cells were seen in any of the biopsy specimens (not shown). In addition, the cells on direct IF that bound anti-IgE did not react with anti-IgG.

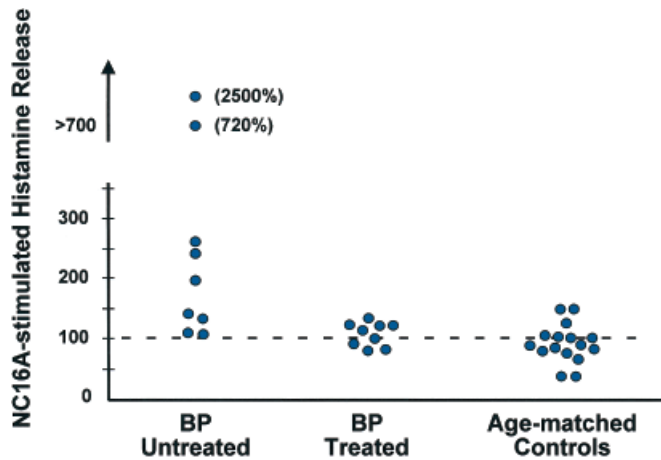


Figure 4. NC16A stimulated histamine release from circulating basophils. Circulating basophils were incubated with 10 ng of NC16A and histamine release was measured by competitive immunoassay. Results with expressed as the percent of background. Untreated BP patients showed a statistically significant release of histamine in response to NC16A when compared with controls ($p = 0.006$). No significant difference was seen in the release from treated BP patients and the control patients. No release above baseline was seen in the 16 age-matched controls.

Basophils from untreated BP patients release histamine in response to exposure to recombinant NC16A As it was not feasible to extract mast cells from BP skin to test directly their degranulation in response to NC16A, histamine release from peripheral blood basophils in response to the NC16A domain of BP180 was measured (Fig 4). The ratio of the NC16A stimulated release to spontaneous release was found to be significantly higher in the untreated BP patients when compared with the control population (2.51 vs 0.872, $p = 0.0006$) and with the treated patients (2.51 vs 1.031, $p = 0.01$). No significant difference was seen in the release by treated BP patients and controls (1.031 vs 0.872, $p = 0.29$). The untreated BP patients were marginally older than the controls ($p = 0.07$); however, there was no relationship between age and histamine release ratios.

Of the nine untreated patients, seven showed an increase in histamine release above baseline in response to NC16A. Five of the nine untreated patients that were tested for histamine release had IgE directed against NC16A detected by immunoblotting. All five of these patients showed increased histamine release in response to NC16A. In addition, two patients in whom we were unable to detect NC16A-specific IgE by blotting techniques also showed some release when challenged with NC16A. Interestingly, one of the untreated patients that showed no histamine release above spontaneous with NC16A did not react with the NC16A domain of BP180 by enzyme-linked immunosorbent assay or western blot. This patient does, however, react with the entire BP180 protein extracted from normal human keratinocytes, suggesting that this patient's serum recognizes a region of BP180 other than the NC16A domain.

DISCUSSION

The classic role for IgE class antibodies in the immune response is in allergic and parasitic diseases. More recently a role for IgE in autoimmune disease has been postulated but its part in autoimmunity has not been elucidated. Most work regarding IgE in autoimmunity thus far has been on the role of autoantigens in atopic dermatitis and in animal models, such as the flaky skin mutant mouse and mercury-induced autoimmunity in rats (Pelsue *et al*, 1998; Seiberler *et al*, 1999; Gorrie *et al*, 2000). Interestingly, IgE class autoantibodies against thyroid peroxidase have recently been identified in patients with Graves' disease and

Hashimoto's thyroiditis (Guo *et al*, 1997; Sato *et al*, 1999), but their pathogenic significance remains unknown. Our study provides further evidence that IgE may be involved in the development of human organ-specific autoimmune diseases.

Several findings now implicate IgE class autoantibodies in the pathogenesis of BP. In this study we found that 70% of the untreated BP patients had elevated total IgE levels, a number that is similar to that reported by other authors (Arbesman *et al*, 1974; Bowszyc-Dmochowska *et al*, 2000). We also showed that these IgE autoantibodies react with the same antigenic target as that of the IgG class autoantibodies. Further, the IgE of most BP patients reacted with the same region of the BP180 protein, NC16A, that has been shown to be of pathogenic relevance with IgG class autoantibodies. Dendritic cells can express the FcεRI under certain disease conditions and may utilize this receptor to present IgE-defined antigens to T cells (Kraft *et al*, 1998). Autoantibodies against the FcεRIα have also been identified in 20% of BP patients (Fiebiger *et al*, 1998). In contrast to the anti-BP180 IgE antibodies we have identified, these IgG autoantibodies did not cause histamine release from mast cells (Fiebiger *et al*, 1998). The way in which these autoantibodies against the IgE receptor may contribute to the pathogenesis of BP is unknown.

The presence of IgE autoantibodies in BP is particularly interesting in light of the fact that BP patients typically have an early urticarial phase of the eruption. During this time the patients have large urticarial plaques but may manifest few if any blisters. Sequential histologic examination of the skin of BP patients during the development of individual lesions has shown that mast cell degranulation is an early event followed by eosinophil migration into the lesion (Wintroub *et al*, 1978; Dvorak *et al*, 1982). The role of IgE-mediated degranulation of mast cells in allergic forms of urticaria is well established (Friedmann, 1999). Wheals of short duration are caused by histamine, whereas the involvement of other mediators may produce eruptions of longer duration. In addition, mast cell degranulation and release of tryptase can cause separation of the epidermal-dermal junction experimentally (Kaminska *et al*, 1999).

In contrast to a previous report, we rarely found IgE at the BMZ by direct IF. This older report used polyclonal antibodies against IgE, and their lack of reactivity with other immunoglobulin classes was not demonstrated (Provost and Tomasi, 1974). As indirect IF identified some patients with circulating IgE capable of binding the BMZ, the negative results on standard direct IF most likely represent a lack of sensitivity compared with salt-split indirect IF.

Skin biopsies of our untreated BP patients showed IgE coating the surface of dermal mast cells. Moreover, many of these mast cells also labeled with a monoclonal antibody against BP180, indicating that BP180 or fragments thereof are bound to the surface of dermal mast cells in BP. This suggests that BP180-specific IgE coating the surface of mast cells results in their degranulation when exposed to BP180 peptides. Cleavage of BP180 into smaller polypeptides is part of its normal processing (Schacke *et al*, 1998; Hirako, 1998; Schumann *et al*, 2000) and may provide a source of BP180 peptides to the mast cells. Alternatively, disruption of the BMZ by IgG binding may cause release of antigen. Whereas it was not possible to test directly the response of patients' skin mast cells to BP180, the fact that circulating basophils from untreated BP patients released histamine in response to recombinant NC16A suggests that anti-BP180 IgE is capable of binding to cells that express FcεRI on their surface.

These data support the possibility that IgE class antibodies play a part in the pathogenesis of BP. Acting in concert with IgG class antibodies they may produce the complete spectrum of the BP phenotype. Further work with purified IgE is planned to identify more directly its role in BP.

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