

A Pathogenic Role for IgE in Autoimmunity: Bullous Pemphigoid IgE Reproduces the Early Phase of Lesion Development in Human Skin Grafted to nu/nu Mice

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Bullous pemphigoid (BP) is an autoimmune disease characterized by subepidermal blistering. Based on previous work, IgG autoantibodies directed against BP180 are thought to be the primary pathogenic agent in BP. In addition to these IgG autoantibodies, however, most BP patients produce IgE class autoantibodies that also react with BP180, and total IgE levels are often elevated in this disease. To directly test whether BP IgE is pathogenic, 6 ng of total IgE isolated from two BP and two normal sera were injected into human skin grafted onto athymic, nude mice. Twenty-four hours after injection, erythematous, elevated plaques were observed in all human skin grafts receiving BP IgE ($n=11$), but not control IgE ($n=9$). Histologic and ultrastructural examination of the lesions showed engorgement of blood vessels and a dermal infiltrate composed of neutrophils, eosinophils, and mast cells, many of which were degranulated. At a higher dose of BP IgE (47 ng), histological separation of the epidermis from the dermis was observed in two of the three grafts. The BP IgE-induced erythematous plaques were reminiscent of those clinically seen in BP. This provides early evidence of a direct demonstration of a pathogenic role for IgE class autoantibodies in a human autoimmune disease.

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INTRODUCTION

Bullous pemphigoid (BP) is an acquired blistering skin disease characterized by a humoral autoimmune reaction directed against components of the hemidesmosome, a cellular structure involved in anchoring the epidermis to the dermis. The initial phase of lesion development in BP is characterized by urticarial plaques consisting of dermal edema accompanied by eosinophilic infiltration of the upper dermis, termed eosinophilic spongiosis. As the lesions progress, there is clinical development of tense, fluid-filled vesicles, which correspond histologically to a separation of the skin through the basement membrane zone (BMZ). In addition, there is a superficial and deep perivascular infiltrate of many eosinophils, lymphocytes, mast cells, and neutrophils.

The seminal work of Beutner and Jordon (Jordon *et al.*, 1967), in which immunoglobulins and C3 were shown to be deposited along the cutaneous BMZ of BP patients, pointed to

the immunological basis of this disease. These investigators were also the first to identify anti-BMZ antibodies in the sera of BP patients. The primary antigenic targets of BP autoantibodies were subsequently identified as two hemidesmosomal proteins, BPAG1 (Stanley *et al.*, 1981) and BP180 (also termed collagen XVII and BPAG2) (Labib *et al.*, 1986; Diaz *et al.*, 1990; Giudice *et al.*, 1992). BPAG1 is a component of the intracellular hemidesmosomal plaque (Tanaka *et al.*, 1991), and its role in pathogenicity remains unknown. In contrast, numerous studies using *in vivo* and *in vitro* models of BP strongly suggest that antibodies directed against BP180 are of primary pathogenic importance (reviewed in Liu and Diaz 2001). Injection of IgG antibodies against BP180 into neonatal mouse skin initiates an inflammatory reaction resulting in skin fragility at the level of the BMZ; however, urticarial plaque formation, recruitment of eosinophils and gross clinical blistering are not seen. Likewise, the injection of human BP IgG into human skin transplanted onto SCID mice leads to histological subepidermal separation, but no clinical disease that mimics BP or any eosinophilic infiltration (Zillikens *et al.*, 2001).

The majority of the experimental work on BP has focused on IgG class autoantibodies, even though it has been long known that there is also an IgE response associated with this disease (Provost and Tomasi, 1974). 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, and the role of IgE-mediated degranulation of

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Abbreviations: BP, bullous pemphigoid; BMZ, basement membrane zone
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mast cells in allergic forms of urticaria is well established (Friedmann 1999). During this early phase of BP, patients have large urticarial plaques but may manifest few, if any, blisters. Sequential histological examination of the skin of BP patients during the development of individual lesions has shown that mast cell degranulation is the earliest event that can be identified histologically, followed by lymphocytic infiltration and later eosinophil and neutrophil migration into the lesion (Wintroub *et al.*, 1978; Dvorak *et al.*, 1982). Further evidence for mast cell degranulation is the presence of eotaxin (Shrikhande *et al.*, 2000) and tryptase (D'Auria *et al.*, 2000) in the blister fluid of BP lesions. Also of note, mast cell tryptase has been shown experimentally to be capable of causing epidermal-dermal separation (Kaminska *et al.*, 1999).

Total circulating IgE levels are elevated in 70% of untreated BP patients (Dimson *et al.*, 2003, Arbesman *et al.*, 1974), and IgE class autoantibodies directed against BP180 can be detected in 90% of untreated BP patients (Dimson *et al.*, 2003). Moreover, basophils isolated from the peripheral blood of untreated BP patients were shown to degranulate when exposed to recombinant BP180 (Dimson *et al.*, 2003). The important implication of this finding is that IgE autoantibodies specific for BP180 are present at the surface of basophils, bound to the IgE receptor. We have hypothesized that an analogous process is occurring in mast cells in the skin of BP patients, and that autoantigen-induced mast cell degranulation is a key trigger for the urticarial phase of BP. To test the pathogenic potential of IgE class autoantibodies in BP, we isolated IgE from BP patients' sera and injected it into human skin transplanted to athymic nude (nu/nu) mice.

RESULTS

Levels of total IgE are elevated in BP sera

Total serum IgE levels were determined by a standard capture ELISA. The IgE concentrations in sera BP-W7714 and BP-G5554 were 18 and 4 $\mu\text{g/ml}$, respectively. Compared to the upper limit of the normal range for serum IgE (240 ng/ml), the IgE levels for BP-W7714 and BP-G5554 are 75 and 17-fold higher, respectively. Western blot analysis revealed that both BP sera contained IgE that reacts with the NC16A domain of type XVII collagen (see below).

Purified BP IgE continues to bind to NC16A, the BMZ, and mast cells

IgE was purified from sera BP-W7714 and BP-G5554 using a combination of negative and positive selection (see Materials and Methods). This purification scheme yielded approximately 10,000-fold purification of IgE relative to IgG. The IgE and IgG concentrations in both of the purified BP IgE preparations were 350–500 and 10–25 ng/ml, respectively. The IgE continued to bind to the BMZ of human skin and cutaneous mast cells, as determined by immunofluorescence using species-specific FITC-conjugated anti-IgE (Chemicon, Temecula, CA) (Figure 1a). The purified IgE also retained its ability to bind to BP180 by immunoblotting (Figure 1b).

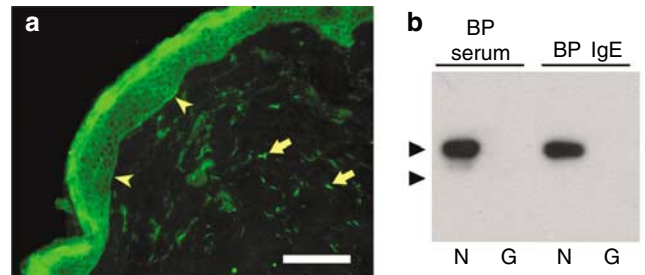


Figure 1. Purified BP IgE binds to basal epidermal keratinocytes and dermal mast cells by IF, and to the NC16A region of BP180 by immunoblotting.

Following purification, IgE from serum BP-W7714 was tested by indirect immunofluorescence for its ability to bind to components of human skin. (a) A cryosection of normal human skin was labeled with the purified BP IgE followed by FITC-anti-human IgE. Staining can be seen at the BMZ (arrowheads), as well as on some dermal cells (arrows). (b) This is an immunoblot of GST-NC16A fusion protein and GST (designated "N" and "G", respectively) labeled with either the unfractionated BP-W7714 serum (left two lanes) or IgE purified from BP-W7714 (right two lanes). The secondary antibody used for all four lanes was horseradish peroxidase-anti-human IgE. The two arrowheads to the left of the blot indicate the positions of GST-NC16A (upper) and GST (lower). IgE reactivity with the GST-NC16A fusion protein is seen in the first and third lanes. Identical results were seen with BP-G5554. Bar = 100 μm .

Injection of BP IgE produces urticarial plaques and mast cell degranulation in human skin grafts

The model system that was used to test the pathogenicity of BP IgE involved grafting human skin onto the backs of nude mice. Cryosections of 2-week grafts exhibited normal labeling patterns with antibodies to human BP180, laminin 5, and collagen VII, thus showing the integrity of the BMZ (data not shown). Retention of human mast cells in the grafts was verified by staining with Giemsa stain and anti-human mast cell tryptase antibodies (Chemicon, Temecula, CA; data not shown).

IgE purified from BP-W7714 and BP-G5554 was injected intradermally into the 2-week human skin grafts. Injection volumes were kept constant at 100 μl . Within 15 minutes after injection of 6 ng of BP IgE, edema was noted within the grafted skin. At 24 hours, an edematous, erythematous plaque developed in all of the grafts that received IgE from either of the BP sera ($n=11$, Figure 2a and c). When the higher amount of BP IgE (47 ng, $n=3$) was injected into the graft, erythema developed around the injection site within 5 minutes, becoming much more intense at the 15-minute time point. No swelling or erythema was noted in any of the control grafts that received 6 ng of IgE isolated from normal human serum ($n=9$, Figure 2b and d) or in the surrounding murine skin. To test whether the residual IgG remaining in the BP IgE preparation was responsible for the observed changes, we injected 1.25 ng of purified BP-W7714 IgG (approximately 4 to 5 times the amount of IgG present in the 6 ng dose of BP IgE) into the human skin graft. The purified BP IgG produced no clinical or histological evidence of disease (data not shown). These results are summarized in Table 1.

Hematoxylin and eosin staining of the lesional grafts showed congestion of the blood vessels in the upper dermis

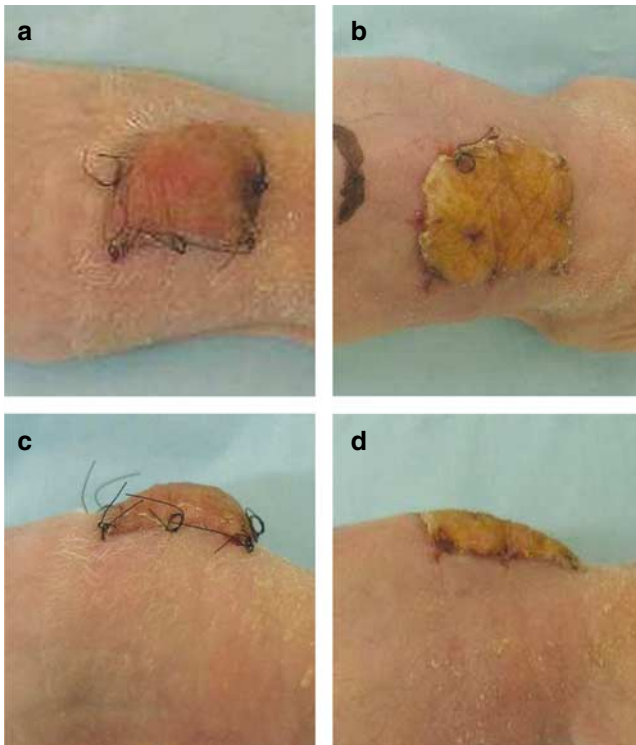


Figure 2. Human skin grafts injected with BP or control IgE. (a and c) Grafts injected with 6 ng of BP IgE show a well-developed urticarial plaque (11/11). (b and d) No elevation or erythema was seen, the control grafts injected with 6 ng of normal IgE.

Table 1. Summary of passive transfer experiments

Injection	No. of mice	Inflammatory plaque
BP IgE, 6 ng	11	11/11
BP IgE, 47 ng	3	3/3 ¹
BP IgG, 1.25 ng	4	0/4
Control IgE, 6 ng	9	0/9
Control IgE, 47 ng	3	0/3

BMZ, basement membrane zone; BP, bullous pemphigoid.

¹Two of the three grafts injected with 47 ng of BP IgE developed histological separation at the BMZ.

and a granulocytic infiltrate, which varied from sparse to more dense near the margins of the grafts (Figure 3a and b). The denser infiltrate was generally seen near the margins of the grafts, where we had previously noted an increased number of mast cells on Giemsa stain. At the higher dose of IgE, microscopic separation at the BMZ was seen in two of the three grafts (Figure 3c). The histological level of the blister was mapped by indirect immunofluorescence using BPAG1- and collagen VII-specific antibodies. BPAG1 and collagen VII labeling were detected in the ceiling and floor of the IgE-induced vesicle, respectively (Figure 3e and f). This indicated that the level of the split was in the lamina lucida, the same site as lesional BP. Direct immunofluorescent staining showed weak focal deposition of human IgE at the BMZ of

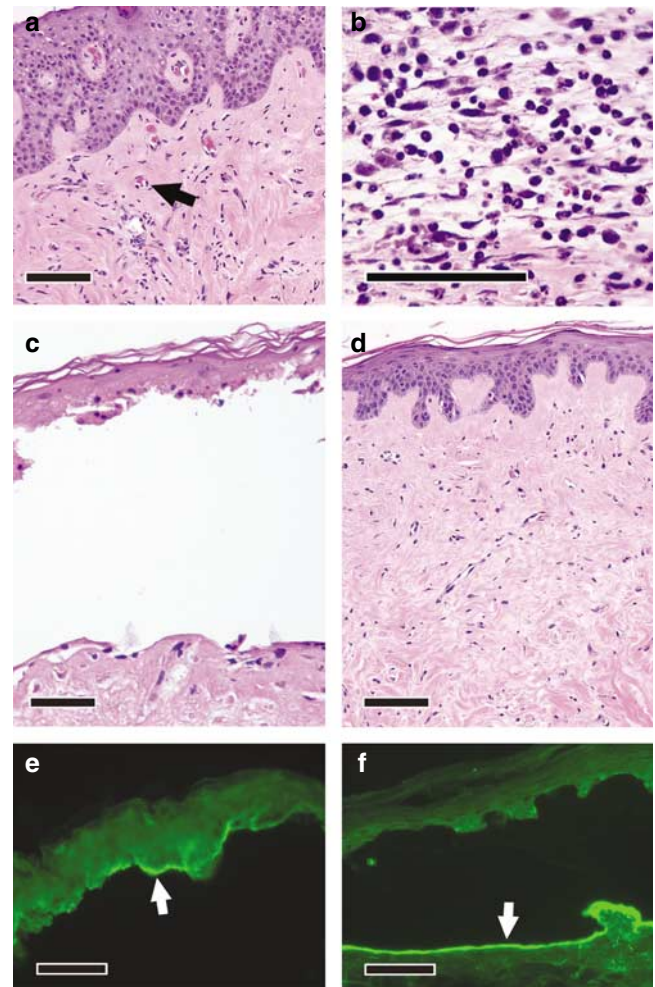


Figure 3. Histology of skin grafts injected with BP IgE. (a) The low-power view of a human skin graft injected with 6 ng of BP IgE. Mild congestion of the vessels is seen (arrow) and a granulocytic infiltrate. (b) Area of dense infiltration showing the granulocytic character of the infiltrate. (c) Two of the three human skin grafts receiving 47 ng of BP IgE split along the basement membrane zone. (d) Control grafts that received normal IgE show no abnormalities. Immunofluorescent mapping with antibodies to (e) BPAG1 and (f) type VII collagen indicated the split was predominantly through the lamina lucida/lamina densa region. Bar = 100 μ m.

the graft and stronger staining on mast cells (Figure 4a and b). No staining was seen with anti-human IgG or anti-murine C3 (Figure 4c and d). Electron microscopy identified mast cells within the lesional region of the grafts (Figure 5a). Many of the mast cells contained empty granules, indicating recent degranulation. In addition, eosinophils, neutrophils, and lymphocytes were also identified in the infiltrate (Figure 5b and c).

DISCUSSION

Historically, the primary role for IgE class antibodies was in the immune response in allergic and parasitic diseases. Recently, a role for IgE and mast cells has been proposed in the regulation of immune responses and in the development of autoimmune disease (Frossi *et al.*, 2004). This hypothesis is

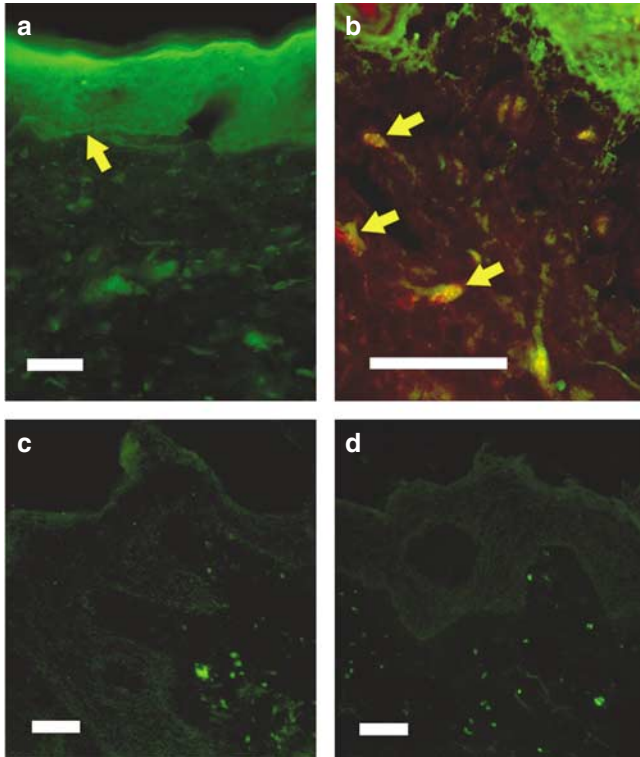


Figure 4. IgE, IgG, and C3 labeling in grafts injected with BP IgE. Following harvest of the grafts 24 hours after injection with 6 ng of BP IgE, cryosections were labeled with FITC-conjugated anti-human IgE, IgG or anti-murine C3. (a) Anti-human IgE revealed weak staining at the BMZ. (b) Mast cells were also labeled by anti-IgE as indicated by double labeling with anti-human mast cell tryptase. (c and d) No BMZ staining was seen with anti-human IgG or anti-murine C3. Bar = 50 μ m.

supported by the recent identification of IgE class autoantibodies in a number of autoimmune diseases, including Grave's disease and Hashimoto's thyroiditis (Guo *et al.*, 1997, Sato *et al.*, 1999), 3); however, the pathogenic relevance of these IgE's remains unknown. Elevated levels of IgE have also been demonstrated in anti-SSA-positive women with lupus 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 the organ-specific autoimmune disease, 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).

In this report, we showed that injection of BP IgE into human skin grafts resulted in lesions that recapitulated the clinical features of the urticarial lesions of BP, including the presence of degranulated mast cells and eosinophils. In early BP, the patients may have large urticarial plaques but manifest few, if any, blisters. Sequential histological examination of the skin of BP patients during the development of individual lesions has shown that mast cell degranulation is the earliest event followed by migration of lymphocytes and later eosinophils and neutrophils into the lesion (Wintroub

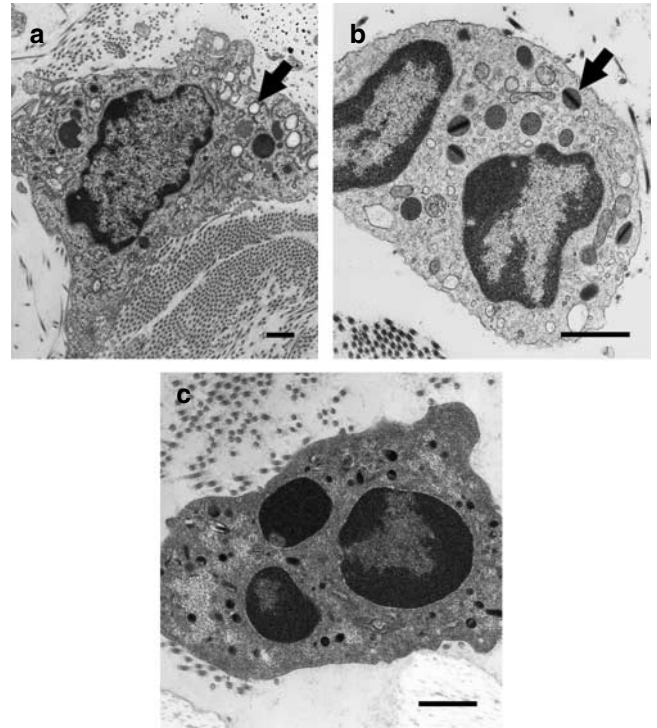


Figure 5. Electron micrographs of the infiltrate induced by BP IgE. The infiltrate was composed of (a) mast cells, many of which have empty granules (arrows), indicating recent degranulation. (b) Eosinophils, recognized by their characteristic granules and bilobed nucleus, were also present, as were (c) neutrophils and lymphocytes (data not shown). Bar = 1 μ m.

et al., 1978; Dvorak *et al.*, 1982). Further evidence for mast cell degranulation is the presence of eotaxin in the blister fluid of BP lesions (Shrikhande *et al.*, 2000).

The level of IgE used in these studies (60 ng/ml) approximates the level of IgE present in normal human sera. The higher level (470 ng/ml) is slightly above the normal range of IgE, but approximates that seen in a larger group of our BP patients (Dimson *et al.*, 2003). Thus, the level of IgE that we are testing in these experiments is within the physiologic range.

The IgE isolation procedure used in the present investigation yielded greater than 10,000-fold purification of IgE relative to IgG. The level of total IgG remaining in the BP IgE purified preparations was in the range of 2–3 ng/ml. This means that each 6 ng dose of BP IgE contained roughly 0.3 ng of total IgG. To determine whether this level of IgG could be responsible for the observed effects induced by our IgE injections, we performed the same type of passive transfer experiments using 1.25 ng of IgG purified from serum BP-W7714. This key control demonstrated that BP IgG at this concentration, which corresponds to approximately 4–5 times the amount of IgG present in the 6 ng dose of BP IgE, did not trigger any detectable changes in the skin grafts. This finding is supported by a previous report by Zillikens and co-workers (Zillikens *et al.*, 2001), who injected as much as 400 μ g of purified BP IgG (over 800,000 times greater than the amount of IgG present in our BP IgE preparation) into

human skin grafted onto SCID mice and detected no clinical or histological lesions.

Another important control that we implemented was to purify IgE from normal control sera and inject it into the human skin grafts. This normal IgE was purified using the same protocol that was used to purify BP IgE and was used at the same dose as that used for the BP IgE. The control IgE failed to induce any specific changes in the injected skin grafts. This control rules out the possibility that the observed changes in the skin grafts induced by our BP IgE injections were non-specific effects of IgE or elements resulting from the purification procedure. Based on the results of these controls, it is clear that the changes that we observed in the human skin grafts can be attributed to the IgE from the BP serum.

Although not directly tested in this study, it is probably that the IgE antibodies responsible for the observed pathologic response are directed against the keratinocyte surface protein, BP180, or fragments of it formed by metabolic degradation. It is well established that a high percentage of BP patients produce anti-BP180 autoantibodies of both IgG and IgE isotypes. Our research group has also recently shown that these IgE autoantibodies largely target NC16A, the non-collagenous linker region of this antigen's ectodomain (Fairley *et al.*, 2005). Furthermore, antigen-specific degranulation of circulating basophils from BP patients can be elicited by treatment with the NC16A peptide (Dimson *et al.*, 2003). We therefore hypothesize that the mechanism for the pathogenic reaction observed in our present human skin graft model involves the binding of the injected BP IgE to the IgE receptor on the surface of human dermal mast cells, and degranulation of these cells after binding of the surface-bound IgE to its antigenic target, thus releasing histamine and other inflammatory mediators. The relatively weak staining for IgE at the BMZ in the BP IgE-injected grafts when compared to the staining seen on mast cells would support this hypothesis. The stronger staining seen on the mast cells may be due to higher affinity of the IgE for its receptor on mast cells than for the BMZ.

It is known that a large stable fragment of the BP180 ectodomain is shed from the keratinocyte surface (Schacke *et al.*, 1998; Schumann *et al.*, 2000). This 120 kDa shed form could well serve as the antigenic activator in this pathogenic process. The rapidity with which we saw a reaction (less than 5 minutes in the high-dose grafts) coupled with the electron microscopy findings strongly support mast cell degranulation as the mechanism for the early-phase lesion development in the grafts. In addition to amplifying the inflammatory reaction, the activated mast cells also release proteases that could well contribute directly to dermal-epidermal separation.

Another possible component of the BP IgE-mediated response may involve the binding of IgE to BP180 on the surface of basal keratinocytes. This binding could impair BP180 function directly, or, more likely, initiate a pro-inflammatory signaling cascade. One concern with this scenario is that IgG would "out compete" for the available binding sites on BP180, diminishing the importance of IgE autoantibodies. Another is that the staining for IgE seen in our

model was weak compared to that on mast cells. 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 material utilized in this study, the concentrations of circulating BP180-specific IgE and IgG were similar (6–9 and 24 $\mu\text{g/ml}$, respectively; unpublished observations), although the relative avidities were not measured. More work is needed to determine the plausibility of this type of IgE-mediated effect on keratinocytes in the pathogenesis of BP.

Complement fixation at the BMZ, a characteristic immunopathologic finding in BP, was one feature absent in our BP IgE-injected grafts. Since IgE does not activate complement, this was not unexpected. Clearly, IgE alone is not capable of replicating all of the pathologic features of BP. Previous work from our laboratory and others point to IgG class autoantibodies directed against BP180 as the trigger for complement fixation at the BMZ and for the subsequent recruitment of inflammatory cells involved in dermal-epidermal separation (Liu *et al.*, 1993, 1995; Chen *et al.*, 2002; Sitaru *et al.*, 2002). Just as in the case of IgE, it is also clear that IgG alone cannot account for all of the pathologic features of BP. Conspicuously absent from the IgG passive transfer model are the key changes associated with the urticarial stage of BP. It is highly likely that the clinical and immunohistochemical manifestations we see in patients represent the effects of both IgE and IgG class autoantibodies.

In a previous report, we demonstrated that the fine specificities exhibited by the IgG and IgE class autoantibodies are quite similar, and thus are likely to have arisen via clonal selection (Fairley *et al.*, 2005). After an initial humoral immune response, which is typically IgM, cytokine-mediated DNA recombination events can occur leading to a switch in the isotype, while largely maintaining the antigen-binding properties. Subtle changes in the binding affinity to the target antigen can occur during isotype switching via somatic hypermutation of the complementarity determining region (Chaudhuri and Alt, 2004). Owing to the chromosomal positions of the γ and ϵ genes, a class switch from IgG to IgE is possible, while the reverse is not. Production of IgE requires the cytokines IL-4 and -13 as well as ligation of CD40 to CD40L on B cells (reviewed in Prussin and Metcalfe, 2003).

The findings of this study represent early evidence pathogenicity of IgE autoantibodies. We showed that injection of BP IgE into human skin grafts resulted in lesions that produced urticarial plaques characterized by the presence of degranulated mast cells. The degranulation of mast cells recapitulated the earliest events of lesion development reported by previous investigators (Wintroub *et al.*, 1978; Dvorak *et al.*, 1982). The presence of pathogenic IgE autoantibodies may be indicative of the type of initial trigger which leads to this disease or the manner in which the antigen is presented to the immune system. BP provides a unique opportunity to study further the role of IgE in autoimmunity. These findings may also open up new areas for potential therapeutic intervention in BP or other human autoimmune diseases.

Addendum: While this paper was under review, Nishie *et al.* (2007) have published an additional murine model of BP. This model utilizes human BP IgG in a humanized mouse that expresses the human BP180 protein. Detachment of the epidermis was seen in animals injected with BP IgG; however, like other models, the urticarial plaques and eosinophilia characteristic of BP were absent.

MATERIALS AND METHODS

Characterization of IgE and IgG in BP and normal sera

This study was approved by the Human Research Review Committee of the Medical College of Wisconsin Affiliated Hospitals (no. 230-01) and the VA Medical Center and was performed in adherence to the Declaration of Helsinki Principles. Consent was obtained from all patients. Levels of total IgE and IgG were measured by capture ELISA (Bethyl Labs, Montgomery, TX).

Purification of BP IgE and BP IgG

IgE was purified from BP sera BP-W7714 and BP-G5554 using a multistep process modified from Kleins-Tebbe *et al.* (1995). Serum was diluted 1:1 in phosphate-buffered saline and the IgG was removed by multiple passes over a protein G affinity column (Pierce, Rockford, IL). By indirect immunofluorescence, the titers of IgG directed against the BMZ went from 1:2,560 and 1:1,280, respectively, in the starting sera to undetectable following the protein G column. The resulting IgG-depleted fraction was incubated with an ϵ -chain-specific monoclonal anti-human IgE coupled to Affi-Gel HZ or Affigel-10 (Bio-Rad, Hercules, CA) and eluted with 25 mM triethylamine pH 11.2 with 1% BSA, and immediately neutralized. The IgE-containing eluate was extensively dialyzed against sterile phosphate-buffered saline and concentrated. BP IgG was purified by affinity chromatography utilizing a protein G affinity column (Pierce). The IgG was eluted with 0.1 M glycine pH 2–3 and immediately neutralized with 1 M Tris pH 8.0. The IgG was extensively dialyzed against sterile phosphate-buffered saline (pH 7.4). The IgG and IgE concentrations were determined by ELISA (Bethyl Labs).

Injection of BP IgE into human skin grafts

These experiments were approved by Animal Studies Subcommittee (VA2655-02P). A 2 × 2 cm area of skin was removed from the back of 6-week-old nu/nu mice. A similar sized piece of human skin obtained from reduction mammoplasties was grafted onto the back of the mice. After 14 days, the expression of BMZ proteins in the graft was tested by indirect immunofluorescence utilizing antibodies directed against collagen VII (gift of Drs Mei Chen and David Woodley, University of Southern California), laminin 5 (gift of Kim Yancey, Medical College of Wisconsin) or BP180 (Liu *et al.*, 1995). The presence of mast cells in the graft was determined with Giemsa stain, and their human origin was established by direct IF utilizing anti-human mast cell tryptase (Chemicon, Temecula, CA) and techniques as described previously (Dimson *et al.*, 2003).

One hundred microliters of BP IgE (60 ng/ml, $n = 11$ or 470 ng/ml, $n = 3$) was injected into 14-day human skin grafts on nu/nu mice. Controls included normal human IgE purified by our laboratory in the same manner as BP IgE ($n = 9$). Non-specific human IgE (<http://www.diatec.com>, Oslo, Norway) was also tested at the high concentration, 47 μ g per graft ($n = 3$). The commercial IgE was used for the more concentrated injection, since concentration of the IgE

we isolated from normal sera would also have increased the concentration of any contaminants from the purification scheme that could affect our injections. The grafts were harvested 24 hours after injection and fixed for routine histology, direct immunofluorescence, and electron microscopy. Electron microscopy was performed in the Electron Microscopy Facility at the Medical College of Wisconsin.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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