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FcR-Independent Effects of IgE and IgG Autoantibodies in Bullous Pemphigoid

Kelly N. Messingham,* Rupasree Srikantha,* Amy M. DeGueme,[†] and Janet A. Fairley*[‡]

Bullous pemphigoid (BP) is a subepidermal blistering disease characterized by IgE and IgG class autoantibodies specific for 180-kDa BP Ag 2 (BP180), a protein involved in cell-substrate attachment. Although some direct effects of BP IgG have been observed on keratinocytes, no study to date has examined direct effects of BP IgE. In this study, we use primary cultures of human keratinocytes to demonstrate Ag-specific binding and internalization of BP IgE. Moreover, when BP IgE and BP IgG were compared, both isotypes stimulated FcR-independent production of IL-6 and IL-8, cytokines critical for BP pathology, and elicited changes in culture confluence and viability. We then used a human skin organ culture model to test the direct effects of these Abs on the skin, whereas excluding the immune inflammatory processes that are triggered by these Abs. In these experiments, physiologic concentrations of BP IgE and BP IgG exerted similar effects on human skin by stimulating IL-6 and IL-8 production and decreasing the number of hemidesmosomes localized at the basement membrane zone. We propose that the Ab-mediated loss of hemidesmosomes could weaken attachment of basal keratinocytes to the basement membrane zone of affected skin, thereby contributing to blister formation. In this article, we identify a novel role for IgE class autoantibodies in BP mediated through an interaction with BP180 on the keratinocyte surface. In addition, we provide evidence for an FcR-independent mechanism for both IgE and IgG class autoantibodies that could contribute to BP pathogenesis. *The Journal of Immunology*, 2011, 187: 000–000.

Bullous pemphigoid (BP) is an autoimmune blistering skin disease characterized by a humoral reaction directed against components of the hemidesmosome, a cellular structure involved in anchoring the basal keratinocytes of the epidermis to the dermis. This reaction leads to the influx of immune cells and formation of tense fluid-filled blisters, which correspond histologically to separation of the epidermis from the dermis through the basement membrane zone (BMZ). An immunologic basis for BP was first suggested by the discovery of anti-BMZ Abs in patient sera and deposition of Igs and complement (C3) along the BMZ in biopsied skin (1). BP disease severity has been shown to be correlated with circulating levels of autoantibodies that recognize the epidermal protein, 180-kDa bullous pemphigoid Ag 2 (BP180, also termed type XVII collagen or BPAG2) (2–5). Through the use of in vitro and in vivo models of BP, it has been established that BP180-specific autoantibodies are the primary pathogenic factors in this disease (6–9). Within BP180, the immunodominant epitopes associated with BP have been mapped to the extracellular linker domain termed noncollagenous 16A (NC16A) (10–14). Approximately 90% of BP sera have both IgG

and IgE class autoantibodies specific for the NC16A region of BP180 (12, 15–18).

Mouse models involving the passive transfer of IgG class anti-BP180 Abs replicate some, but not all, of the key features of BP. Although inflammation and skin fragility at the level of the BMZ were observed in the IgG passive transfer models, other hallmarks of BP that were absent included urticarial plaque formation, recruitment of eosinophils, and spontaneous subepidermal blistering (6, 7, 14). These shortcomings of the IgG-based models led to investigations into the role of IgE class autoantibodies in the BP disease process (17, 19–21). Passive transfer of IgE autoantibodies, purified from patient sera, into human skin grafts on nude mice was shown to induce lesions in the grafted skin that reproduce the early phases of BP lesion development, including mast cell degranulation, eosinophil influx, formation of erythematous plaques, and histological separation at the BMZ (19). Zone and colleagues (21) made similar observations after s.c. injection of a murine IgE hybridoma specific for the shed ectodomain of human BP180 (LABD97) in SCID mice with engrafted human skin.

A wide variety of human and mouse models has been used to determine the mechanism(s) of autoantibody-induced blister formation in BP. Findings from IgG passive transfer murine models have demonstrated that complement activation (22), mast cell degranulation (23), and neutrophilic infiltration (24) are required for separation at the BMZ. The translation of these findings to human disease is difficult because of species-dependent differences in FcR expression and function (25–27). Another type of model has been used to address this that involves the incubation of BP autoantibodies with cryosections of normal human (NH) skin. These studies demonstrated that NC16A-specific IgG, granulocytes, and FcγRII and FcγRIII are all necessary to achieve a subepidermal split (9, 28, 29). Furthermore, the successful treatment of a BP patient, who was resistant to standard therapies, with an Ab that prevents IgE binding to FcεRI has defined a role for this receptor in BP (20). Together, these findings show that development of clinical BP is dependent on FcR engagement;

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Abbreviations used in this article: BP, bullous pemphigoid; BP180, 180-kDa bullous pemphigoid Ag 2; BMZ, basement membrane zone; BP IgE, IgE purified from the serum of a patient with BP; BP IgG, IgG purified from the serum of a patient with BP; C IgE, commercially available nonspecific human IgE; DIF, direct immunofluorescence; NC16A, noncollagenous 16A domain of BP180; NH, normal human; NHEK, normal human epithelial keratinocyte; NH IgE, IgE purified from normal human serum; NH IgG, IgG purified from normal human serum; TEM, transmission electron microscopy.

however, they do not exclude an additional mechanism that is independent of FcR binding.

A limited number of studies have examined the direct effects of autoantibody binding using primary cultures of human keratinocytes. Schmidt and colleagues (30) found that addition of IgG purified from the serum of a patient with BP (BP IgG) resulted in a dose-dependent increase in the production and secretion of IL-6 and IL-8 (30), cytokines known to be elevated in the sera and tissues of BP patients (31, 32) and critical for blister formation in mouse models (24). Moreover, addition of BP IgG to cultured keratinocytes led to internalization of immune complexes (33) and decreased adhesive strength (34). To date, no studies have demonstrated any downstream effects of Ag-specific binding of IgE purified from the serum of a patient with BP (BP IgE).

In this report, we confirm BP IgE binding to the BMZ of perilesional skin in vivo and use primary cultures of human keratinocytes to demonstrate the downstream effects of direct binding and internalization of BP IgE. A comparison of the direct effects of BP IgE to BP IgG on cultured keratinocytes and human skin organ cultures provides further evidence that FcR-independent and -dependent effects of both autoantibody isotypes may contribute to lesion development in BP.

Materials and Methods

Sera, tissue, and Ab purification

Serum samples were collected from patients who met the clinical, immunologic, and histologic criteria for BP: subepidermal blistering and direct immunofluorescence (DIF) demonstrating IgG and/or C3 at the BMZ of perilesional skin. Control sera were obtained from age- and sex-matched patients with no known autoimmune disease. Written informed consent was obtained before inclusion in the study. Deidentified archival blocks of lesional or perilesional skin biopsies from BP patients were obtained from the University of Iowa Department of Pathology. This study was approved by the Institutional Review Board at the University of Iowa and was performed in adherence to the Declaration of Helsinki Guidelines.

IgE was purified from BP and NH sera using a multistep process as described previously (19). This purification scheme removes 99.9% of IgG from the IgE preparation and the concentration of contaminating IgG is added to control wells. The purified autoantibodies retain their reactivity to the NC16A domain of BP180 by ELISA and bind to the BMZ of skin (18, 19). Because of the difficulty in purifying sufficient amounts of IgE from controls, some experiments used a commercially available nonspecific human IgE (C IgE; Assay Designs, Ann Arbor, MI). Ab Fab and F(ab')₂ fragments were generated using kits with immobilized papain or pepsin, respectively, according to the manufacturer's instructions (Pierce, Rockford, IL).

Keratinocyte culture, cell treatment, and MTT

Primary cultures of NH epithelial keratinocyte (NHEK) obtained at p2 (Cascade Biologics, Portland, OR) and immortalized keratinocytes that do not express BP180 (BP180 null; gift of Kim B. Yancey, University of Texas Southwestern) (35) were maintained below confluence in keratinocyte serum-free media (Life Technologies, Carlsbad, CA).

For experiments, cells were plated directly into the wells (2×10^4 cells/well), or onto 12-mm glass coverslips (1×10^4) placed in the wells, of 24-well tissue culture plates and incubated overnight at 37°C. The next day, fresh media containing IgE (BP or NH) or IgG (BP or NH) was added to duplicate cultures at the concentrations indicated and incubated as described above. Coverslips were processed for confocal or electron microscopy (see later). Supernatants were collected and frozen at -20°C until analysis. The total number of viable cells per well was estimated using the MTT assay as directed by the manufacturer (Sigma, St. Louis, MO).

Skin organ culture

Replicate 2-mm punch biopsies were excised from neonatal foreskin tissue obtained from routine circumcision (36). The punch biopsies were incubated for 24 h (4/well) in a 96-well tissue culture plate in a total volume of 0.2 ml keratinocyte serum-free media with or without IgE or IgG Abs (NH or BP) at concentrations noted in the figure legends. Organ culture-conditioned medium was collected and frozen at -80°C for cytokine

ELISA, and tissue specimens were frozen in OCT for cryosection or processed for transmission electron microscopy (TEM).

Cytokine detection

Cytokine production was evaluated as part of a multiplex assay (Bio-Rad Bioplex) as directed and analyzed using Luminex at the University of Iowa Flow Cytometry Core or using commercially available ELISA kits specific for human IL-6 and IL-8 (R&D Systems).

Immunofluorescence

Tissue-bound autoantibodies were detected at the BMZ of using DIF on serial cryosections (5 µm) using FITC-conjugated anti-human IgG or IgE (Bethyl Laboratories, Montgomery, TX) (17). Slides were viewed with an epifluorescent Nikon photomicroscope.

Flow cytometry

Cells were detached nonenzymatically, washed, and resuspended in FACS buffer (PBS/1% FCS, 0.002% NaN₃). Abs used include HD18 (monoclonal mouse IgG specific for the NC16A region of human BP180) (35), anti-mouse IgG-FITC, anti-human IgE-FITC or anti-human IgG-FITC, or isotype controls (Bethyl Laboratories, Montgomery, TX). Whether IgE or IgG present in BP sera could bind keratinocytes was determined after incubation of cell suspensions with a 1:10 dilution of a BP or control serum for the indicated times.

Cell lysate and immunoblot

Lysates were prepared as described previously (37), and expression of BP180 was determined by immunoblot using a polyclonal Ab (R136) specific for BP180 (38). To confirm equal loading, membranes were stripped and reprobed with an anti-GAPDH Ab (Abcam, Cambridge, MA).

Confocal microscopy

The localization of bound IgE was evaluated using anti-human IgE-FITC and standard protocols (39). Coverslips were mounted, and nuclei counterstained, using Prolong-gold antifade with DAPI (Invitrogen, Eugene, OR). Confocal images were taken using a Bio-Rad Radiance 2100MP Multiphoton/Confocal Microscope at the University of Iowa Central Microscopy Research Facility and analyzed with ImageJ (National Institutes of Health).

Transmission electron microscopy

Cells grown on coverslips were fixed and processed according to standard TEM protocols (6). Images were recorded using a JEOL JSM1230 TEM equipped with a Gatan USC1000 2Kx2K CCD camera.

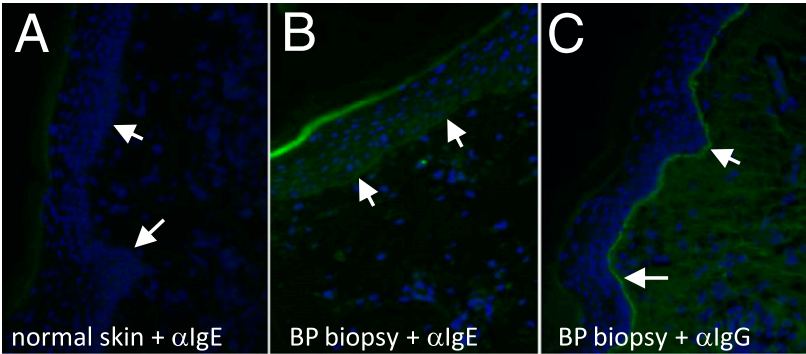
Statistics

Statistical significance was determined using a one-way ANOVA followed by a Mann-Whitney *U* test for post hoc analysis, and $p \leq 0.05$ was considered significant. Data were analyzed using GraphPad Prism Statistical Software (GraphPad, San Diego, CA).

Results

The hallmark of BP is the linear deposition of IgG and/or C3 at the BMZ of biopsied lesional or perilesional skin. Although an early report indicated that ~30% of biopsies also have IgE deposition at the BMZ, this has not been confirmed (40). To examine this, we incubated cryosections from seven BP biopsy specimens and four control samples with FITC-conjugated Abs specific for human IgG or IgE and counterstained cell nuclei with DAPI. Representative images are shown in Fig. 1. As expected, the majority of BP biopsies (5/7) showed bright linear staining at the BMZ (indicated by arrows) when IgG-specific secondary Abs were used for detection (Fig. 1C). Similar deposition of IgE was detectable in three of seven BP biopsies (Fig. 1B); however, in contrast with IgG, staining was less intense and often discontinuous. In all cases where IgE was detectable at the BMZ, IgG was also found (Table I). No specific labeling was detectable when NH skin sections were incubated with IgG- (data not shown) or IgE-specific (Fig. 1A) Abs, or when BP biopsies were incubated with isotype control Abs (data not shown).

FIGURE 1. Both IgE and IgG class autoantibodies are deposited at the BMZ of skin in BP. In vivo autoantibody deposition was evaluated by DIF on cryosections (6 mM) of lesional/perilesional BP biopsies or normal skin using anti-human IgE- or IgG-FITC (green) and DAPI nuclear stain (blue). The BMZ is indicated by arrows. Linear deposition of both IgE (B) and IgG (C) is evident. Neither IgE (A) nor IgG (data not shown) was detectable in normal skin. Photos are representative staining from seven BP and four control samples. Original magnification $\times 400$.



Deposition of autoantibodies at the BMZ is evidence of direct Ag binding by both isotypes in vivo. Despite the fact that these autoantibodies are specific for BP180 (11, 12), it has not been definitively shown that BP IgE can act independent of the FcεRI and exert direct effects on keratinocytes through Ag–Ab interaction. To address this, we used flow cytometry to verify the expression of BP180 on the surface of NHEK. Next, BP IgE binding to the surface of NHEK was evaluated after incubation (30 min, 4°C) with BP serum. In both cases, BP180-null keratinocytes were used as controls. As expected, BP180 was readily detectable ($>88\%$) on the surface of NHEK, but not BP180-null keratinocytes (Fig. 2A), and BP IgE binding was detectable on $>95\%$ NHEK, but not BP180-null keratinocytes (Fig. 2B). The fluorescence intensity of BP IgE-labeled NHEK was diminished with serum dilution or by trypsinization of cells before staining (data not shown). BP180 expression, or lack thereof, was verified in NHEK and BP180-null keratinocytes by immunoblot (Fig. 2C). To determine whether both IgE and IgG from BP serum could simultaneously bind to BP180 expressed on the surface of NHEK, we incubated cells at 37°C with BP sera, and both BP180 expression and autoantibody binding were monitored over time (0.5, 1, and 2 h). Surface expression of BP180 decreased over time, which could be because of Ag blocking effects of BP sera or internalization of BP180 (Fig. 2D, left panel). Similarly, both autoantibody isotypes were initially detectable on NHEK, but the staining intensity decreased over time. Incubation with the same

BP sera for 0.5, 1, and 2 h resulted in 59, 30, and 0% positive for IgE and 88, 67, and 43% positive for IgG, respectively (Fig. 2D, center panels). As expected, autoantibody binding was not detectable on BP180-null keratinocytes incubated with BP sera (Fig. 2D, right panel). Next, Ab internalization was confirmed using confocal microscopy. The cellular localization of purified BP IgE was visualized over time (15 and 60 min) after cells were treated with BP IgE at a concentration similar to that observed in the circulation of BP patients (60 ng/ml). Punctate IgE staining that localized to the cell surface was visible 15 min after Ab addition (Fig. 3A). Within 60 min of BP IgE addition, the majority of staining appeared to be cytoplasmic (Fig. 3B). Examination of the z-series projection (vertical slice) associated with each image confirms internalization of the bound IgE as the staining moves from the periphery to the cytoplasm (Fig. 3A, 3B). No staining was visible if cells were

Table I. Both IgG and IgE are bound to the BMZ of biopsied skin in BP

Patient No. ^a	IgG	IgE
4529	—	—
1337	++	+
5714	++	—
7298	—	—
2770	+	+
1459	+	+
4579	+	—
Total	5/7	3/7
Control 1 ^b	—	—
Control 2	—	—
Control 3	—	—
Control 4	—	—
Total	0/4	0/4

Autoantibodies were detected as weakly positive (+), strongly positive (++), or negative (—) compared to isotype controls using FITC-conjugated Abs specific for human IgG or IgE.
^aDeidentified lesional or perilesional biopsy specimens were obtained from patients with a confirmed diagnosis of BP.
^bDeidentified discarded skin from was obtained from surgical procedures.

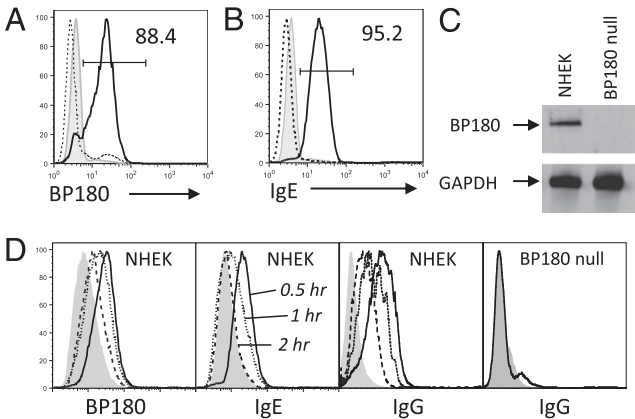
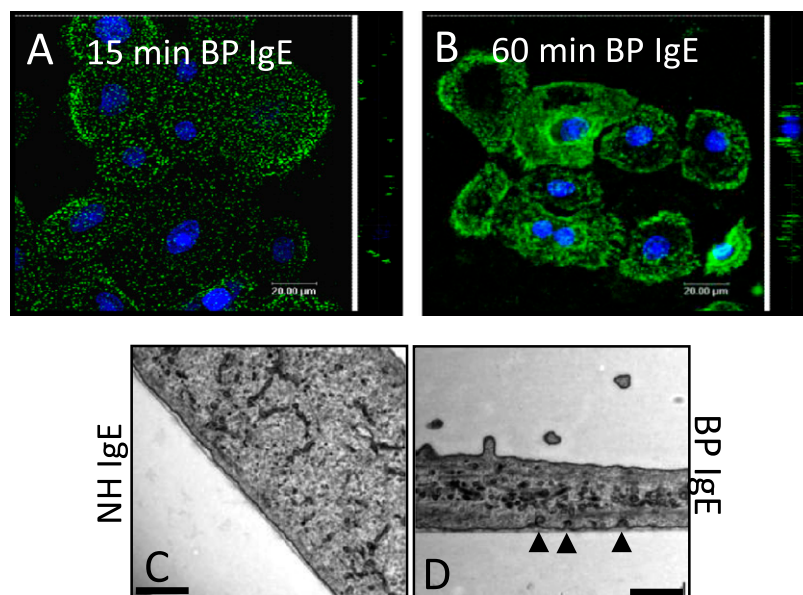


FIGURE 2. Ag-specific binding of BP autoantibodies decreases surface expression of BP180. A, BP180 expression is detectable by flow cytometry on NHEK (solid line), but not on BP180-null (dashed line) cells, using a BP180-specific mAb (HD18). No staining was visible using isotype control on NHEK (shaded histogram). B, Anti-human IgE staining revealed autoantibody binding to NHEK (solid line), but not BP180-null keratinocytes (dashed line) incubated with BP sera. No staining was visible using isotype control on NHEK incubated with BP serum (shaded histogram). C, Immunoblot using HD18 confirms expression of BP180 on NHEK and its absence on BP180-null keratinocytes. D, Incubation with BP serum alters surface expression of BP180 and the intensity of autoantibody binding over time. NHEK were incubated (37°C) for 0.5 (solid line), 1 (dotted line), and 2 h (dashed line) with BP serum and stained with HD18, anti-human IgE, or anti-human IgG and evaluated by flow cytometry. For all three molecules, the staining intensity decreased over time, compared with appropriate isotype controls (shaded histograms). IgG autoantibody binding was not detectable when BP180-null cells were used (right panel). Panels are representative of three (A, B) or two (C, D) experiments using different BP sera.

FIGURE 3. BP IgE is internalized after binding to the keratinocyte surface. For confocal microscopy, NHEK were cultured in glass chamber slides coated with type IV collagen to ~80% confluence. Purified BP IgE (60 ng/ml) was added to cultures, and after 15 and 60 min, cells were fixed and stained with anti-human IgE-FITC (green) and DAPI nuclear stain (blue). Punctate surface staining typical of hemidesmosomal distribution was observed after 15 min (A) that shifted to intracellular localization by 60 min (B). Scale bars, 20 μ m (A, B). In some experiments, NHEK were grown on glass coverslips and treated with 60 ng/ml BP (C) or NH IgE (D) for 20 h, then processed for TEM. Alterations of the basal membrane (arrowheads) are present after treatment with BP (D), but not control (C) IgE. Scale bars, 0.8 μ m (C, D). Images are representative of two (C, D) or three (A, B) experiments using different preparations of BP IgE.



treated with IgE purified from normal human serum (NH IgE) (data not shown). Evaluation of keratinocyte ultrastructure by TEM provided further evidence for Ab-induced internalization of surface molecules with the appearance of membrane-bound (pinocytotic, arrowheads) vesicles on the basal surface of cells treated with BP IgE, but not NH IgE (Fig. 3C, 3D). Taken together, these studies are the first, to our knowledge, to demonstrate direct effects of BP IgE on keratinocyte cell structure and are in agreement with studies demonstrating BP IgG binding to BP180 and subsequent internalization of both molecules *in vitro* and *in vivo* (33, 34, 41).

It is possible that internalization of BP180 could result in alterations in keratinocyte culture morphology or attachment. To address this, we added NH or BP IgE (60 ng/ml) to wells containing equal numbers of NHEK, and we observed culture confluence using phase-contrast light microscopy. Because BP IgG has also been shown to bind to and cause internalization of hemidesmosomal components (34), NHEK were also treated with NH and BP IgG at the same concentration as IgE and a higher dose (2 mg/ml), in the physiologic range for IgG autoantibodies. After 20 h, both the untreated (Fig. 4A) and NH IgE-treated (Fig. 4B) NHEK had a similar appearance; however, BP IgE treatment resulted in an obvious decrease in culture confluence (Fig. 4C). The addition of BP IgG, compared with IgG purified from normal human serum (NH IgG), also led to a decrease in the confluence of NHEK that appeared to be concentration dependent (Fig. 4D–F) but was less remarkable than the effect observed with BP IgE.

To assess whether the observed decrease in culture confluence resulted from cell detachment and/or cell death, we estimated the total number of cells per well using the MTT assay. Treatment with either BP IgE or IgG decreased the total number of cells per well in comparison with wells treated with their respective control Abs (Fig. 4G).

Others have shown that addition of BP IgG to keratinocyte cultures resulted in increased production and secretion of IL-6 and IL-8 (30, 42). To determine whether BP IgE could similarly stimulate keratinocyte cytokine secretion, we treated cells with 60 ng/ml NH IgE, C IgE, BP IgE, and NH IgG or BP IgG for 20 h and assayed supernatants (Fig. 5A, 5B). BP IgE treatment of keratinocytes resulted in a significant ($p < 0.01$) increase in IL-6 and IL-8 compared with treatment with either of the control IgE Abs or medium (diluent) alone. BP IgG treatment of keratinocytes

stimulated a significant ($p < 0.05$) increase in IL-6 production, although this was dramatically less than observed with BP IgE at the same concentration. No significant increase in IL-8 production was observed when NHEK were treated with 60 ng/ml BP IgG. When physiologic (2 mg/ml) levels of BP IgG were used, a dramatic ($p < 0.001$) increase in IL-8 production by NHEK was observed in comparison with all other treatment groups (Fig. 5C). Furthermore, treatment of NHEK with F(ab')₂ and Fab fragments prepared from BP IgE (60 ng/ml) or BP IgG (2 mg/ml) confirm that FcR engagement is not involved in the keratinocyte cytokine response, but that the valency of the Ab is important (Fig. 5D).

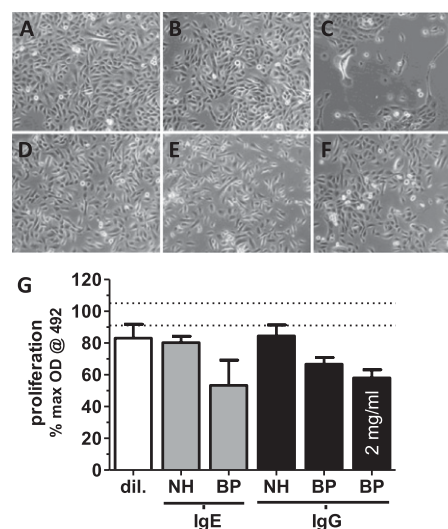


FIGURE 4. Treatment with BP autoantibodies influences keratinocyte culture morphology. NHEK cultures were observed using phase-contrast microscopy (original magnification $\times 200$) after incubation (20 h) of triplicate cultures with autoantibodies purified from BP sera as indicated: BP IgE (C, 60 ng/ml) or IgG (E, 60 ng/ml; F, 2 mg/ml). As controls, NH IgE (B, 60 ng/ml), NH IgG (D, 2 mg/ml), or an equivalent volume of diluent (A; dil.) was also added. Images are representative of three experiments using different sources of BP IgE. Whether autoantibody treatment influenced cell viability was addressed using the MTT assay after incubation of NHEK cultured for 24 h with IgE (NH or BP), IgG (NH or BP), or dil. G, Graph depicts one of two independent experiments in which two to three sources of BP IgE and BP IgG were tested in duplicate.

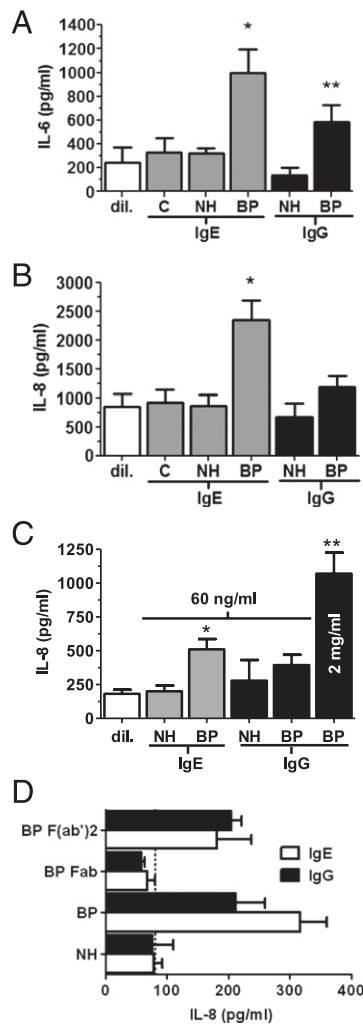


FIGURE 5. BP IgE and IgG stimulate the production of IL-6 and IL-8. *A* and *B*, NHEK (5×10^4) were treated for 20 h with 60 ng/ml BP or NH IgE, a C IgE, or BP or NH IgG. An equivalent volume of diluent (dil.) was also used as a control. Cell free supernatants were collected for cytokine analysis (*A*, *B*). * $p < 0.05$ versus NH or C IgE or dil., ** $p < 0.05$ versus NH IgG. *C*, Addition of 2 mg/ml BP IgG results in increased secretion of IL-8 by NHEK, compared with 60 ng/ml of either isotype. * $p < 0.01$ versus other IgE, ** $p < 0.05$ versus other IgG. *D*, NHEK treatment with F(ab')₂ fragments of BP IgE did not significantly affect IL-8 secretion; however, treatment with Fab fragments resulted in a loss of IL-8 induction. Experiments using intact IgE and IgG were conducted a minimum of three times, and F(ab')₂ or F(ab) experiments were done twice.

Primary cell cultures are ideal to dissect the direct effects of antibody addition on keratinocyte biology; however, they are limited in their ability to tell us whether any of the observed changes impact epidermal architecture or adhesion. The human organ culture is valuable as a readout for studies investigating mechanisms of other autoimmune blistering diseases because the architecture of human skin is maintained (43, 44). For our purposes, the absence of infiltrating immune cells is an added benefit. In this study, human organ cultures were used to determine the effect(s) of BP autoantibody treatment on intact human skin. Biopsy punches were maintained for 24 h in organ culture in the presence or absence of IgE or IgG Abs (NH or BP). Cryosections of treated biopsy punches were stained using immunofluorescence to ensure diffusion and binding of BP autoantibodies to autoantigens. As expected, deposition of both IgE and IgG is visible at the BMZ using IgE- or IgG-specific detection Abs after incubation

of skin with either isotype of BP autoantibodies (Fig. 6*B*, 6*D*, filled arrows). No BMZ staining was visible with NH IgE or IgG (Fig. 6*A*, 6*C*, arrowheads).

Thus far, our data suggest that binding of BP IgE to BP180 on the surface of cultured keratinocytes results in alterations in both culture morphology and ultrastructure at the basal surface, as well as internalization of surface-bound autoantibodies. Similarly, BP IgG has been reported to deplete cultured keratinocytes of BP180 and weaken cell attachment (34, 39), most likely by disturbing the formation of hemidesmosomes (34). To visualize autoantibody effects on the ultrastructure of skin organ cultures treated with physiologic concentrations of NH or BP IgE (200 ng/ml) or NH or BP IgG (2 mg/ml), we examined the BMZ using TEM. Samples from two independent experiments are shown in each row. In all cases, the junction at the BMZ is clearly visible with the plasma membrane of the basal keratinocytes and the basal lamina of the dermis bordering the electron-lucent lamina lucida (Fig. 7, double asterisks) (6). In organ cultures treated with NH IgE (Fig. 7*A*) or IgG (Fig. 7*C*), the thickened hemidesmosomal attachment plaques (indicated by arrows) were readily visible along the BMZ. In contrast, the hemidesmosomes observed in organ cultures treated with BP IgE (Fig. 7*B*) or IgG (Fig. 7*D*) were fewer and appeared rudimentary. As expected, this observation was not dependent on FcR engagement, as treatment of organ cultures with F(ab')₂ fragments of either isotype produced similar results.

Supernatants collected from skin organ cultures treated with IgE (NH or BP) and IgG (NH or BP) were assayed for IL-6 and IL-8 (Fig. 8). Treatment with BP IgE resulted in a significant increase in the production of both cytokines, whereas treatment with BP IgG stimulated a significant increase in IL-8 production by skin organ cultures, but only a marginal increase in IL-6.

Discussion

It is well established that IgG autoantibody deposition at the BMZ plays a critical role in the local activation of complement (C3) and the FcR-dependent recruitment and activation of immune cells in

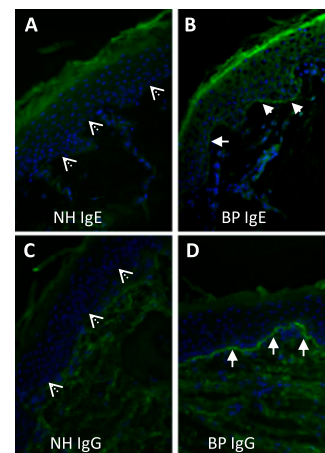


FIGURE 6. BP IgE and IgG bind the BMZ of skin organ cultures. Autoantibody deposition was evaluated by DIF using anti-human IgE- or IgG-FITC (green) and DAPI nuclear stain (blue) on cryosections (5 μ M) of skin organ cultures derived from 2-mm punch biopsies of human foreskin. Punches (4/well) were incubated for 24 h in a 96-well plate in 0.2 ml medium containing IgE (NH or BP, 200 ng/ml) or IgG (NH or BP, 2 mg/ml). The BMZ is indicated by arrows. Linear deposition (filled arrows) of both IgE (*B*) and IgG (*D*) is evident at the BMZ when punches were incubated with BP autoantibodies, but not with control Abs (*A*, *C*, arrowheads indicate BMZ). Images are representative of two (IgG) or three (IgE) independent experiments conducted using autoantibodies prepared from different BP sera. Original magnification $\times 400$.

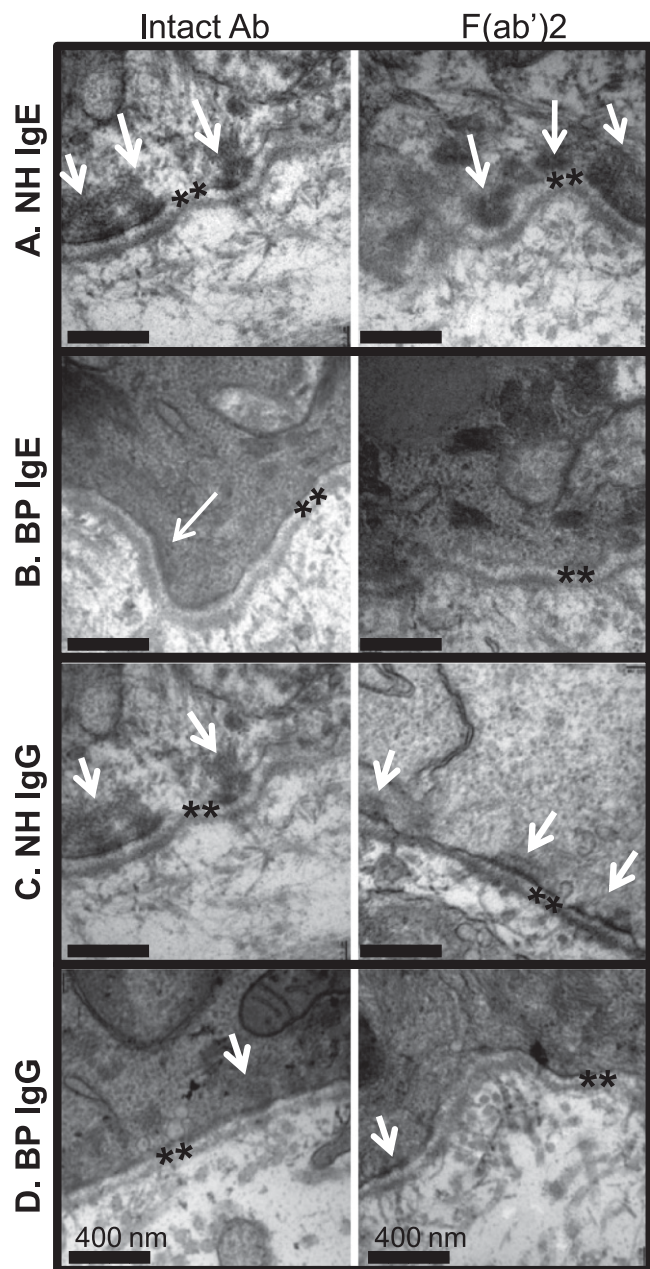


FIGURE 7. Both BP IgE and IgG reduce hemidesmosomes at the BMZ in organ cultured skin. Biopsy punches (2 mm) were processed for TEM after incubation (24 h) in intact Abs or F(ab')₂ fragments of IgE (NH or BP, 200 ng/ml) or IgG (NH or BP, 2 mg/ml). All images are oriented with epidermis on top. The lamina lucida (**) is visible between the plasma membrane of the basal keratinocytes and the lamina densa of the dermis. Hemidesmosomes (arrows) were readily visible in NH IgE- (A) or NH IgG-treated (C) samples, but were reduced in number when BP IgE (B) or IgG (D) was used. Similar results were obtained when F(ab')₂ Ab fragments were used. One representative image from two (IgG) or three (IgE) independent experiments is shown for each treatment. Scale bars, 400 nm.

BP (24, 45, 46). More recently, it has been accepted that the FcεRI-dependent effects of IgE autoantibodies are also necessary to fully recapitulate human disease (19–21). However, specificity of these BP autoantibodies suggests an additional mechanism of action, through direct binding of BP180 on basal keratinocytes. Indeed, FcR-independent effects of BP IgG have been demonstrated using cultured keratinocytes (30, 33, 34, 39). In this report, we demonstrate that BP IgE is capable of Ag-specific binding to

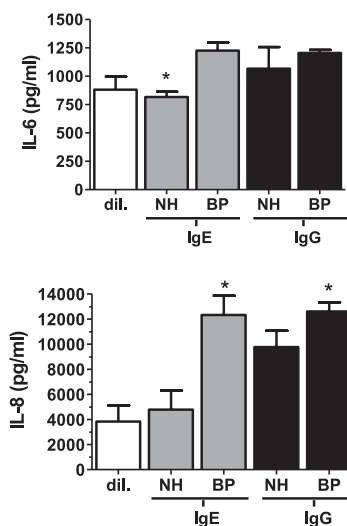


FIGURE 8. BP IgE and BP IgG trigger similar levels of cytokine production in organ cultured skin. Conditioned medium was collected after incubation (24 h) of biopsy punches (2 mm) in IgE (NH or BP, 200 ng/ml) or IgG (NH or BP, 2 mg/ml). Cytokines were assayed by ELISA. Graphs depict IL-6 and IL-8 concentrations in supernatants collected from two (IgG) or three (IgE) independent experiments. **p* < 0.05 compared to BP IgE for IL-6 or compared to the isotype control for IL-8.

keratinocytes in vitro and in vivo, and demonstrate that the FcR-independent effects of BP IgE and BP IgG are similar in both cultured keratinocytes and human skin organ cultures.

The proinflammatory cytokines IL-6 and IL-8 have been implicated in the disease process of BP. In passive transfer models using IgG, the presence of both cytokines at the lesion site is critical for blister formation (24), and the concentration of these cytokines in patient blister fluid is correlated with number of skin lesions (47, 48). This correlation suggests local production of these cytokines within the affected skin. Schmidt and colleagues (30–32) reported a dose-dependent increase in IL-6 and IL-8 production by cultured keratinocytes treated with BP IgG. In this article, we demonstrate that both BP IgE and BP IgG stimulate keratinocyte cytokine production. In our hands, BP IgE appears to be a more potent inducer of cytokine production. Because of technical limitations, the maximal concentration of BP IgE that we are typically able to achieve is <1 μg/ml. Because BP IgG must be used at a concentration ≥1000-fold higher to effect cellular changes, a titration experiment directly comparing both isotypes is not feasible. We propose that this discrepancy is due to a difference in the proportion of BP180-specific Ab for each isotype. Preliminary semiquantitative Western blot analysis of BP sera suggests that a large proportion (30–40%) of the circulating IgE is specific for BP180, whereas a much lower fraction (<1%) of the total IgG is BP180 specific. Thus, the majority of our studies were conducted with physiologic concentrations of BP IgE (ng/ml) or BP IgG (mg/ml). Our confirmation of an earlier report of IgE deposition at the BMZ of perilesional skin (1) and our ability to detect both IgE and IgG on the surface of cultured keratinocytes after incubation with BP sera refute the idea that the much higher concentrations of BP IgG would “outcompete” direct binding of keratinocytes in vivo.

To our knowledge, our laboratory provided the first evidence of the pathogenicity of BP IgE Abs in a model using human skin grafted onto nude mice (19). In these studies, injection of BP IgE (60 or 470 ng) into the grafts resulted in infiltration and degranulation of resident human neutrophils and eosinophils. Microscopic separation at the BMZ was observed in two of three grafts treated

with 470 ng BP IgE. Ideally, we would evaluate the direct effects of BP autoantibodies by comparing intact and F(ab')₂ fragments of BP autoantibodies in this human graft model; however, technical limitations prevent us from producing sufficient amounts of F(ab')₂ fragments to use different doses in multiple mice. Thus, we tested the direct effects of BP IgE and IgG on human skin using an organ culture model. Using skin organ cultures, others (44) have reported variability in both background and LPS-stimulated cytokine secretion from biopsies obtained for either the same or different individuals. To minimize this variability, we used foreskins obtained shortly after birth and placed four punches per well. When considered with the primary culture experiments, increased cytokine production in organ cultures confirms that keratinocytes contribute to local cytokine production within the skin in BP. Furthermore, it suggests that at least some of the early events after autoantibody deposition in the skin, such as cytokine secretion and hemidesmosomal remodeling, originate within the skin itself, independent of resident or infiltrating immune cells.

It has been reported by others that treatment of keratinocytes (DJM-1) with BP180-specific IgG mAbs or IgG purified from BP sera resulted in internalization of BP180 (39). Furthermore, BP IgG reduces the BP180 content of hemidesmosomes, resulting in decreased adhesion of cultured keratinocytes to the cell matrix (34). In this article, we demonstrate that both BP IgE and BP IgG result in a loss of hemidesmosomal localization at the BMZ of human skin. In the majority of lesional and perilesional biopsy specimens from BP patients, BP180-specific IgG was found localized to the plasma membrane (41) and internalization of BP180-BP IgG immune complexes was observed in basal keratinocytes, whereas BP180 was detected only on the surface of basal cells in normal skin (33). Taken together, these studies suggest that both in vitro and in vivo, BP180-specific autoantibodies disrupt the formation of hemidesmosomes and decrease cell attachment. This weakened cell attachment could augment the proinflammatory processes associated with BP and contribute to blister formation in vivo.

There is substantial evidence in both human (9, 29) and mouse (14, 45) models that FcγR-dependent immune inflammatory reaction is necessary for the development of subepidermal blistering in BP. The presence of degranulated mast cells within autoantibody-treated human skin grafted onto immunodeficient mice also support a role for FcεRI-mediated effects of IgE class autoantibodies (19, 21). Furthermore, Ag-specific degranulation of circulating basophils from BP patients can be elicited by exposure to the BP180 NC16A peptide, indicating that Ag-specific IgE is bound to their surface in vivo (17).

The idea that inhibition of BP180 expression or interaction with the extracellular matrix could be detrimental to epidermal adhesion is not new. In vivo subepidermal blistering is observed when expression of BP180 is reduced or absent because of gene knockout in mice or mutation in humans with generalized atrophic benign epidermolysis bullosa (6, 49). Van den Bergh and colleagues (50) demonstrated that in cells that lack other hemidesmosomal components, cell matrix attachment is directly related to the amount of BP180 expression. In this study, we demonstrate that BP autoantibodies decrease confluence of cultured keratinocytes and the number of hemidesmosomes localized at the BMZ of organ cultured skin. Our in vitro studies suggest that autoantibody binding triggers internalization Ag–Ab complexes. In similar studies, degradation of Ag–Ab complexes after BP IgG treatment of keratinocytes would explain the decrease BP180 content that is associated with a loss of adhesion to the dish (34). Thus, it is likely that FcR-independent autoantibody effects could contribute to loss of epidermal adhesion in BP.

In summary, to our knowledge, these studies are the first to identify a direct effect of IgE autoantibodies through interaction with autoantigens on the keratinocyte surface. In addition, we establish that both IgE and IgG class autoantibodies exert similar direct effects on both cultured keratinocytes and human skin. These findings provide additional support for an alternative non-inflammatory mechanism(s) of autoantibody-induced tissue damage that could contribute to blister formation in vivo (51). Although we acknowledge that the FcR-mediated autoantibody effects are necessary for the development of lesions with all of the characteristics of BP, it is likely that the direct effects of autoantibody binding to keratinocytes are also relevant to the disease. Currently, more extensive in vivo studies examining the FcR-dependent and -independent effects of both IgE and IgG are under way. These findings could open up opportunities for novel therapeutic targets for BP.

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Disclosures

The authors have no financial conflicts of interest.

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