

IgE Basement Membrane Zone Antibodies Induce Eosinophil Infiltration and Histological Blisters in Engrafted Human Skin on SCID Mice

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Bullous pemphigoid (BP) is characterized by the deposition of IgG in the basement membrane zone, infiltration of eosinophils, and blister formation. The purpose of this study was to evaluate a potential role of IgE basement membrane antibodies in the histological findings of BP. LABD97 is a component of the shed ectodomain of bullous pemphigoid antigen 2. We have developed an IgE hybridoma to LABD97 antigen. This hybridoma was injected subcutaneously in SCID mice with engrafted human skin. A subcutaneous hybridoma secreting IgE antibodies developed. An IgE mouse hybridoma to trinitrophenyl was used as a control. Human grafts and mouse skin were examined grossly over 21 days, histologically, and immunopathologically at day 21 after injection of the hybridoma. A visible subcutaneous tumor developed in 10–14 days. Erythema and intense scratching developed 2–3 days before the tumor in test mice, but not in controls. At day 21, 16/16 test mice developed intense eosinophil infiltration and degranulation of the human mast cells within the grafts and 13/16 developed histological, but not clinically visible, basement membrane blisters. Human skin grafts of control mice and normal mouse skin on the test mice and control mice did not develop any histological abnormalities. IgE antibodies to LABD97 recapitulate the histological inflammatory process seen in BP.

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INTRODUCTION

Bullous pemphigoid (BP) is an autoimmune inflammatory mucocutaneous disease characterized clinically by urticarial plaques, vesicles, and bullae on an erythematous base. Histologically there is a basement membrane zone (BMZ) blister located in the lamina lucida and infiltration of the superficial dermis with eosinophils. IgG and complement deposition is present at the BMZ, and IgG class circulating BMZ antibodies are present (Borradori and Bernard, 2003). The IgG antibodies react to the BMZ antigens BPAg1 (BP230, a 230-kDa hemidesmosomal plaque protein) (Tanaka *et al.*, 1991), bullous pemphigoid antigen 2 (BPAg2) (BP180, type XVII collagen, a 180-kDa hemidesmosomal transmembrane protein) (Diaz *et al.*, 1990; Giudice *et al.*, 1992), and a portion of the shed ectodomain of BPAg2 termed LABD97 (Meyer *et al.*, 1991, 1990).

Although these IgG BMZ antibodies are widely believed to be responsible for the inflammatory mucocutaneous process,

passive transfer of BP serum to mice failed to reproduce these findings (Anhalt and Diaz, 1987). After noting that the putative pathogenic epitopes (NC16A) of human BPAg2 were not present in mouse skin, Liu *et al.* developed an IgG rabbit antibody directed against the NC16a domain of the mouse BPAg2. Passive transfer to neonatal mice produced neutrophil infiltration and a lamina lucida blister, but no eosinophil infiltration was noted. This model does however replicate some of the features of BP, including a grossly visible BMZ blister and deposition of IgG and complement at the BMZ (Liu *et al.*, 1993).

LABD97 is a 97-kDa proteolytic fragment of BPAg2, which was described on the basis of its reactivity with IgA BMZ antibodies from both adult and childhood linear IgA bullous dermatosis (LABD) of the lamina lucida type (Zone *et al.*, 1990, 1996, 1998). IgG antibodies to LABD97 which do not react with intact BPAg2 are also present in the serum of some BP patients (Meyer *et al.*, 1990). The LABD97 epitope of BPAg2 is present in human skin, but not in mouse skin, and this fragment of BPAg2 can be readily extracted from normal human dermis (Zone *et al.*, 1990). We have previously developed IgG and IgA class mouse mAbs to human LABD97 and passively transferred these antibodies to athymic mice bearing human skin grafts (Zone *et al.*, 2004). Intense BMZ deposition of antibodies resulted, but there was minimal inflammation with occasional neutrophils and eosinophils and rare BMZ blisters.

There is some evidence that IgE antibodies may play a role in BP. Increased IgE levels have been identified in 50–70% of

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Abbreviations: BP, bullous pemphigoid; BMZ, basement membrane zone; LABD, linear IgA bullous dermatosis; TNP, trinitrophenyl; BPAg2, bullous pemphigoid antigen 2

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patients with untreated BP (Arbesman *et al.*, 1974; Delaporte *et al.*, 1996; Christophoridis *et al.*, 2000; Bowszyc-Dmochowska and Dmochowski, 2002; Dimson *et al.*, 2003). Fairley *et al.* (2005) recently reported that 8/10 untreated BP patients had IgE antibodies that reacted with intact BPAg2. Four of eight reacted with the NC16A region of BPAg2 whereas four reacted with sites downstream from NC16A. CD23, a low-affinity receptor for IgE expressed on B lymphocytes, was found to be higher in BP patients compared to normals and tended to be higher in patients with more severe disease, suggesting a potential role of IgE in the pathogenesis of BP (Inaoki *et al.*, 2004).

This study was designed to evaluate the role of IgE class antibodies to LABD97 in a mouse model. We developed IgE class mouse hybridomas to the human LABD97 antigen. The hybridoma cells were injected subcutaneously into SCID mice bearing human skin grafts that expressed the LABD97 antigen. In this report, we describe the clinical and histological results that mimic human BP.

RESULTS

Human skin grafts develop erythema at days 8–11 after subcutaneous injection of IgE hybridomas to LABD97

After injection of the hybridoma subcutaneously (day 0) in the SCID mouse bearing a human skin graft, a visible subcutaneous tumor developed in 11–14 days. At days 8–11, before the development of a visible tumor, all 16 of the test mice started to scratch intensely. The graft became visibly erythematous and swollen (Figure 1). Control animals with IgE hybridomas to trinitrophenyl (TNP) ($n=5$) or phenylarsonate ($n=5$) developed subcutaneous tumors but did not scratch and did not develop erythema of the grafted skin.



Figure 1. Mice receiving IgE anti-LABD97 hybridomas develop erythema in human skin grafts. Photographs of SCID mice bearing human skin grafts at day 21 after hybridoma cell injection. The control mouse (left) received a TNP IgE hybridoma and demonstrated no erythema of the graft. The test mouse (right) received an LABD97 IgE hybridoma and developed intense erythema.

Control animals with IgA hybridomas to LABD97 ($n=4$) and IgG hybridomas to LABD97 ($n=5$) developed subcutaneous tumors but did not scratch and did not develop erythema of the grafted skin. Erythema of the mouse skin was not noted in either the test animals or the controls.

Histological, but not clinical, BMZ blisters with eosinophil infiltration develop in human skin but not in mouse skin

Biopsy of the human skin at day 0 revealed a scant superficial perivascular infiltrate of lymphohistiocytic cells but no evidence of neutrophil or eosinophil infiltration. There was also no evidence of interface inflammation at the BMZ and no evidence of antibody or complement deposition. Biopsy of the human skin from the test animals at days 8–11 revealed infiltration of the papillary dermis with eosinophils visible by hematoxylin and eosin staining as well as staining for mouse major basic protein (Figure 2). Eosinophilic spongiosis was noted within the epidermis. The eosinophils were noted to be intact and no evidence of degranulation of the mouse eosinophils was noted. Occasional neutrophils were noted. Early basement membrane blisters were noted in the samples obtained at days 8–10. Harvest of the grafted skin and serial sectioning at days 18–21 revealed basement membrane separation in 13 of the 16 test animals (Figure 2). Evaluation of the human skin grafts in the TNP and phenylarsonate controls failed to reveal any evidence of eosinophil infiltration or BMZ separation at days 8–11 or at days 18–21. Similarly, histological evaluation of the mouse skin in both the test and control animals failed to reveal any evidence of eosinophil infiltration or BMZ separation at days 8–11 or at days 18–21. All final biopsies taken from the two control groups revealed no blistering or other histopathological changes different from the day 0 specimens. The results are summarized in Table 1.

Intradermal injection of hybridoma supernatant into the human skin graft

Intradermal injection of IgE LABD97 hybridoma fluid diluted in phosphate-buffered saline revealed erythema and scratching within 15 minutes in 3/3 IgE LABD97 mice that received 100 ng/50 μ l. Biopsy at 24 hours revealed eosinophil infiltration without vesicle formation and IgE deposited at the BMZ. IgE was noted to be bound to mast cells in the papillary dermis. The three mice receiving IgE TNP revealed no evidence of erythema or scratching at 15 minutes and no eosinophil infiltration at 24 hours. Binding of IgE to mast cells was noted in the dermis in all three animals receiving IgE TNP. This indicates that IgE LABD97 but not IgE TNP is able to produce inflammation in the absence of other systemic factors that may occur in the subcutaneous hybridoma model.

Immunomapping

Immunomapping of the basement membrane blister revealed staining of the roof of the blister with BP serum and the base of the blister with type IV collagen (Figure 3). This indicates the lamina lucida as the location of the blister as is the case in BP.

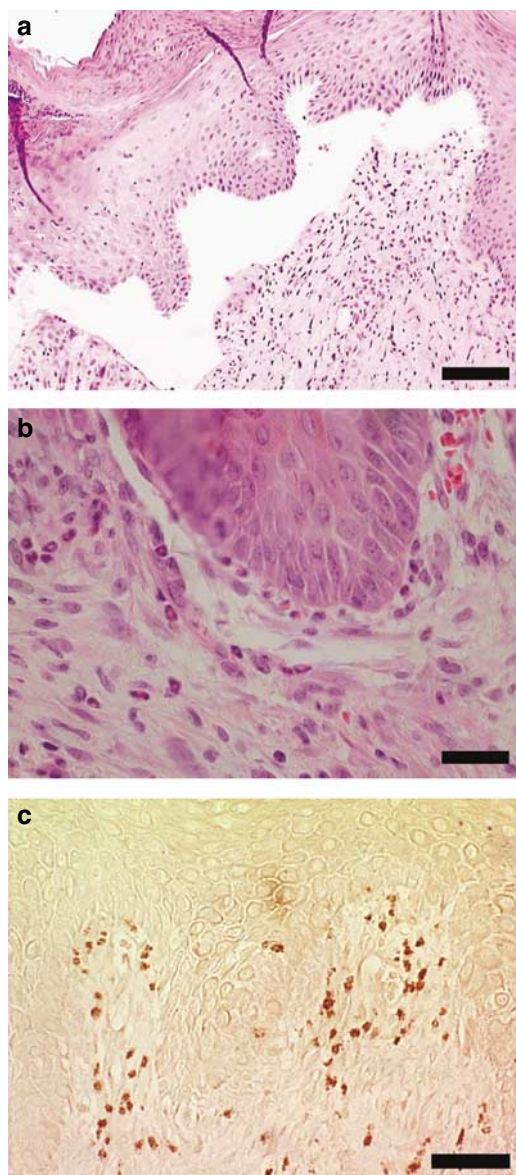


Figure 2. Basement membrane blister and eosinophil infiltration at day 21 in the dermis of a human skin graft of a mouse receiving IgE LABD97 hybridoma. (a) A basement membrane blister is seen at day 21. Bar = 100 μ m. (b) Eosinophils stained with hematoxylin and eosin are seen in the dermis. (c) Eosinophils stained with antibody to major basic protein are seen in the dermis. Bar = 25 μ m.

Control grafts

Goat anti-IgE injected intradermally into the grafts of the TNP and phenylarsonate control mice at days 18–21 produced edema and erythema visually apparent in 10–15 minutes. This served to validate the presence and potential activity of IgE in the control mice.

IgE is present at the basement membrane of test animals

Direct immunofluorescence evaluation of human skin grafts and mouse skin at day 0 failed to reveal any evidence of mouse or human C3, IgG, IgM, IgA, or IgE. This included an absence of mouse IgE on dermal mast cells. Direct immuno-

fluorescence of erythematous skin at days 8–11 revealed intense IgE in a linear pattern along the BMZ when areas without vesiculation were present. In areas of vesiculation IgE staining was either absent or minimally present in the roof of the blister (Figure 4). Staining of both day 8–11 and day 18–21 skin grafts for mouse C3, IgG, IgM, and IgA was negative.

Mouse IgE serum levels in both the TNP and phenylarsonate controls ranged from 125 to 175 μ g/ml whereas IgE levels in the test group ranged from 5 to 12 μ g/ml. The disproportionate ratio seen *in vivo* between the test and control groups mirrors that seen in the growth medium from the hybridomas grown *in vitro*. SCID mice at baseline have no detectable IgE in their serum. However, normal BALB/c mice have levels up to 500 ng/ml (Seitzer *et al.*, 2005). The levels in our test mice are 10–24 times that of normal BALB/c mice. These results are comparable to those published by Dimson *et al.* (2003) for humans with BP. They noted that the mean level of total IgE in untreated human BP patients was 3.3 times the upper limit of the normal with a maximum level of 15 times normal.

The test animals demonstrated serum titers of IgE BMZ antibodies adhering to the epidermal side of BMZ split skin at titers of 1:10 at days 11–14 and negative indirect immunofluorescence at day 21. Once again these numbers are comparable to the data presented by Dimson *et al.* in humans. They noted that 7/23 untreated BP patients had circulating IgE BMZ antibody.

IgA and IgG LABD97 control animals failed to develop inflammation

Biopsy of the IgA and IgG LABD97 control animals revealed no significant infiltration of either neutrophils or eosinophils and no blister formation at day 21. BMZ deposition of IgG and IgA was noted in the IgG and IgA controls, respectively (Table 1).

Human mast cells are present in the graft at day 0, bind IgE, and degranulate during the inflammatory process

At day 0 the human graft stained positive for mast cells with a rhodamine-avidin stain. Mast cells were distributed evenly throughout the papillary and reticular dermis. Mast cells in the graft also stained positive for human tryptase (Figure 5). Mast cells in the mouse skin stained positive with rhodamine-avidin and toluidine blue, but did not stain with the human tryptase antibody (not shown). This indicates that the human mast cells engrafted to the SCID mouse survive during the graft maturation period. However, during the acute inflammatory process at days 8–11, only rare mast cells were visible with the human tryptase stain in the papillary dermis whereas human mast cells continue to be abundant in the reticular dermis. At days 18–21 when mature histological blisters are present, mast cells are again detectable in significant numbers in the papillary dermis. This would seem to indicate that during the acute inflammatory process the mast cells have degranulated and subsequently regenerate their tryptase-containing granules. Human mast cells at baseline fail to show evidence of mouse IgE bound to their surface (not shown). However, at days 18–21 IgE can be detected on the

Table 1. Summary of results of mouse hybridoma experiments

Hybridomas	Experimental animals				Control animals	
	IgE LABD97	IgE LABD97	IgG LABD97	IgA LABD97	IgE TNP	IgE PA
Tissue source	Human graft	Mouse skin	Human graft	Human graft	Human graft	Human graft
Erythema and scratching	16/16	0/16	0/5	0/4	0/5	0/5
BMZ blister	13/16	0/16	0/5	0/4	0/5	0/5
Eosinophils	16/16	0/16	0/5	0/4	0/5	0/5
BMZ IgE	16/16	0/16	0/5	0/4	0/5	0/5
BMZ IgG/C'	0/16	0/16	5/5	0/4	0/5	0/5
BMZ IgA	0/16	0/16	0/5	4/4	0/5	0/5

TNP, trinitrophenyl; PA, phenylarsonate.

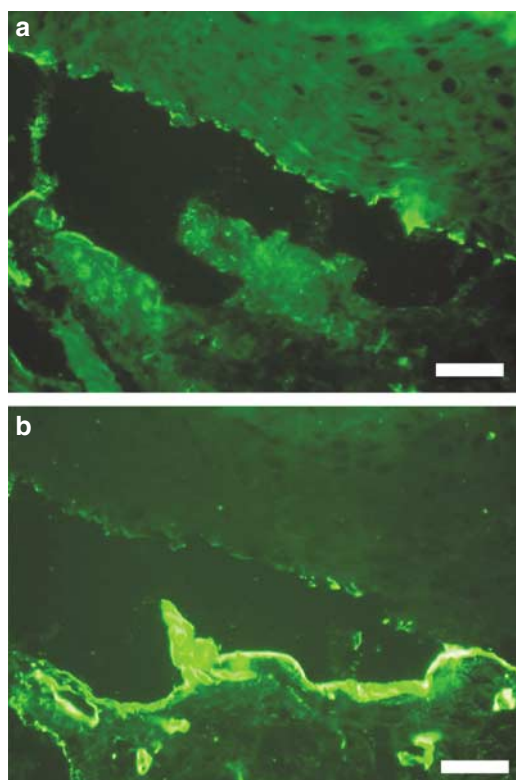


Figure 3. Immunomapping of the basement membrane blister is in the lamina lucida. A BMZ blister from the human graft of a test mouse is stained with antibody to (a) BPAG2 and (b) type IV collagen. This localizes the blister location to the lamina lucida. Bar = 50 μ m.

surface of the mast cells in the graft (Figure 5) and in the normal mouse skin of both test animals and controls (not shown).

The mean decrease in the numbers of mast cells in the test animals comparing the day 8–10 samples with day 0 was 73% whereas in the controls there was an average gain of 100%. Others have also reported an increase in human mast cells in grafted skin with time (Christofidou-Solomidou *et al.*, 1997).

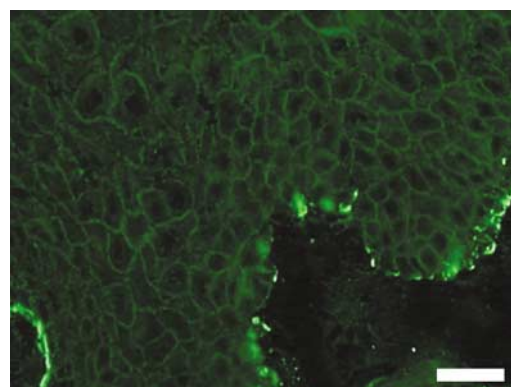


Figure 4. Direct immunofluorescence of a basement membrane blister. The section is stained with fluorescein conjugated anti-mouse epsilon chain. IgE deposition is noted in the roof of the blister. Bar = 50 μ m.

DISCUSSION

We have developed a new animal model that reproduces the clinical and histological findings of human BP. This includes (a) an initial urticarial induration of the human skin associated with scratching, (b) infiltration of the dermis with an infiltrate that is predominantly eosinophils, (c) mast cell degranulation, and (d) basement membrane vesiculation. It should be realized that there is urticarial induration of the skin that contains histological blisters, but no clinically visible blisters. The histological blisters occur in the setting of intense tissue eosinophilia, but we have not shown that the eosinophil is essential in producing these blisters. These characteristics of human BP are produced by the passive transfer of IgE BMZ antibodies alone. In our previous work, passive transfer of IgG and IgA antibodies directed against the same LABD97 antigen failed to produce significant inflammation. The dramatic and highly reproducible findings of this study suggest that there may be a significant role for IgE in the pathogenesis of human BP. The failure of others to develop an animal model that involves eosinophil infiltration may be related to the fact that passive transfer models concentrated on the role of IgG alone (Liu *et al.*, 1993).

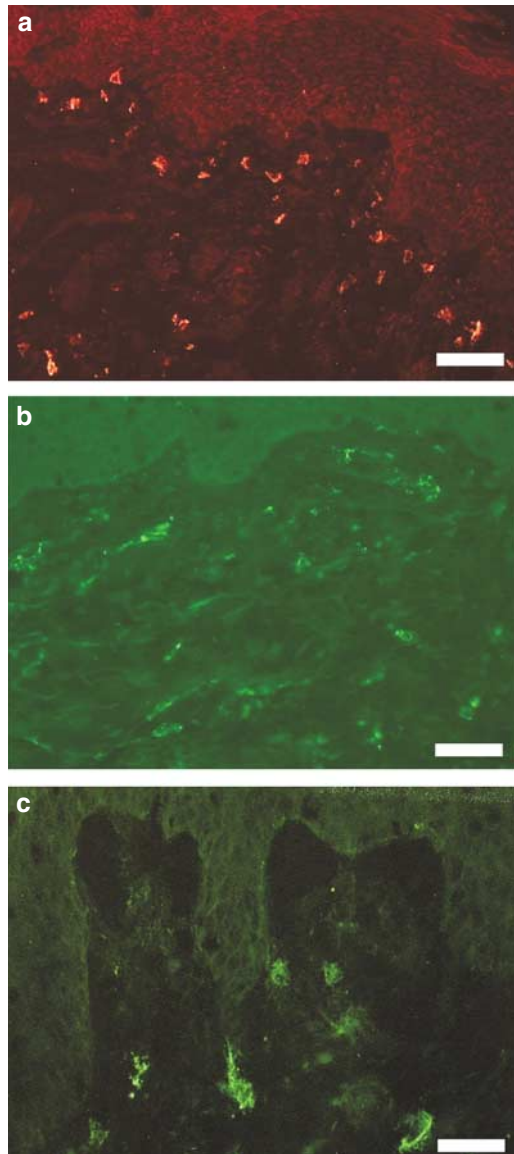


Figure 5. The human grafts contain human mast cells that bind mouse IgE. (a) The human graft at day 0 contains numerous mast cells that stain with avidin. (b) The mast cells also stain with human tryptase indicating that they are of human origin. Mast cells in mouse skin stain with avidin but not human mast cell tryptase (not shown). (c) Mast cells in human skin graft from day 21 stain with anti-mouse epsilon chain. Cells also stain with avidin (not shown). Bar = 50 μ m.

This model utilizes IgE BMZ antibodies directed against the LABD97 antigen and documents the pathogenicity of antibodies to the LABD97 antigen. LABD97 is recognized as a component of the shed ectodomain of BPAG2 (Zone *et al.*, 1998). As such it is shed into the dermis during the normal proteolytic process and can be identified in the dermal extract using standard extraction techniques (Zone *et al.*, 1990). The epitope recognized by our mouse hybridoma antibody is not present in mouse skin (Zone *et al.*, 2004). In this model, antibodies to the antigen are pathogenic in human skin where the antigen is present, but not pathogenic in mouse skin where the antigen is absent. This is a key point

in our theory of pathogenesis in this model, as unbound LABD97 antigen in the papillary dermis is available to crosslink IgE bound to dermal mast cells. Our preliminary work on this model has suggested to us that mast cell degranulation is likely to be an essential component in the evolution of inflammation. Although IgE bound to the mast cells in the normal mouse skin, no inflammation took place. We attribute this to the fact that LABD97 is a human protein that is not present in mouse skin (Zone *et al.*, 2004) and is therefore not available in the papillary dermis for binding to Fab sites and crosslinking IgE anti-LABD97 molecules that are attached to mast cells by Fc binding. In order to prove this hypothesis, it would be necessary to inject purified LABD97 into the mouse skin and evaluate for inflammation and mast cell degranulation. We have not yet performed these experiments, but are in the process of purifying larger amounts of LABD97 for this purpose. In a similar manner, the control TNP and phenylarsonate IgE hybridomas bind IgE to the mast cells in both the human skin graft and the mouse skin of the control animals. However, the TNP and phenylarsonate antigens are not present in the dermis to initiate mast cell degranulation. Moreover, these bound IgE control molecules clearly possess the ability to degranulate the dermal mast cells since the injection of anti-IgE into the graft produces erythema and a wheal and flare response within 10–15 minutes.

Binding of mouse IgE to human Fc epsilon R1 is an essential component of this model. It has been well established that there is *in vitro* cross-species binding of mouse IgE with human basophils, mouse IgE binding with human Fc epsilon R1, degranulation of human basophils by mouse IgE, and subsequent release of enzymes. *In vivo* mouse experiments with subsequent binding of mouse IgE to human mast cells has also been successful (Ishizaka *et al.*, 1985; Hakimi *et al.*, 1990; Haak-Frendscho *et al.*, 1993; Mita *et al.*, 1995; Cho *et al.*, 2000).

It is possible that other local factors produced by the subcutaneous hybridomas contribute to the inflammatory process that is seen in the test mice. If present, these factors were not sufficient to produce inflammation in the mice with IgG, IgA, TNP, or phenylarsonate hybridomas. In the future, we plan to purify large amounts of IgE BMZ antibodies from hybridoma cells and passively transfer this purified antibody as we have done previously for IgA and IgG antibodies (Zone *et al.*, 2004). Preliminary attempts at this have been difficult because of the relatively low amounts of IgE produced by the IgE hybridomas compared to the IgA and IgG hybridomas. We have not been able to produce significant histological inflammation with IgG or IgA hybridomas. It seems likely that if these antibodies are pathogenic, then additional pro-inflammatory factors are required.

We have previously reported the results of passive transfer of IgG and IgA class mouse monoclonal antibodies to LABD97 to athymic mice bearing human skin grafts. This was done by isolating antibody from mouse ascites fluid rather than the current backpack mouse method and subsequently administering the ascites fluid to the athymic mice intraperitoneally (Zone *et al.*, 2004). The passive

transfer of these IgG and IgA antibodies produced high circulating titers of antibody and strong BMZ deposition of antibody, but only a minimal inflammatory infiltrate of eosinophils and neutrophils within the human skin grafts. Microscopic early BMZ blisters without visible graft inflammation were noted in 50% of the animals that received a single IgG class LABD97 monoclonal antibody and 42% of those that received IgA class antibody. The minimal inflammation and vesiculation in these models suggests that these early blisters might have been a result of interference with the structural integrity of the BMZ conferred by BP180 rather than the inflammatory immunologic process believed to be active in human BP. We noted no vesiculation or inflammation in the human grafts from mice bearing IgG or IgA hybridomas in the present experiments. LABD97 antigen is known to be absent in generalized atrophic benign epidermolysis bullosa, the hereditary condition in which the BP180 gene is mutated (Shimizu *et al.*, 1998). This disorder does produce a minimally inflammatory blister related to loss of the structural function of BP180.

Understanding the nature of the LABD97 antigen is central to our theories of pathogenesis. We initially described the LABD97 antigen as the predominant antigen to which serum IgA anti-BMZ antibodies bound from patients with the lamina lucida type of LABD and chronic bullous disease of childhood (Zone *et al.*, 1990; Zone *et al.*, 1996). We also demonstrated that IgG from the serum of BP patients bound LABD97 on immunoblot (Meyer *et al.*, 1990, 1991). Antibodies to the LABD97 antigen did not crossreact with the transmembrane hemidesmosomal BP180 antigen (BPAg2) on immunoblot. However, subsequently using amino-acid sequencing of the immunoaffinity isolated LABD97 antigen, we were able to determine that LABD97 is identical to the extracellular component of BP180. LABD97 localizes on electron microscopy between the NC16A and carboxy terminal domains of BP180 (Ishiko *et al.*, 1998). The lack of cross-reactivity of LABD97 antibodies with BP180 on immunoblot is still not explained. Attempts at binding our LABD97 monoclonal antibodies to various fusion proteins of BP180 have been unsuccessful (unpublished data). We have hypothesized that the LABD97 antigen is a neoantigen that is formed during the proteolytic cleavage of BP180.

Previous studies have strongly implicated a pathogenic role of anti-BPAg2 IgG and neutrophils in BP blister formation and degradation of BPAg2. Verraes *et al.* (2001) noted the presence of neutrophil elastase in skin biopsies of BP patients and recovered active enzyme from blister fluid. They demonstrated that recombinant BPAg2 proteolysis occurred with both neutrophil elastase and metalloproteinase 9, but a specific elastase inhibitor and not an inhibitor of metalloproteinase could only inhibit the pattern of BPAg2 proteolysis induced by BP blister fluid. Sitaru *et al.* (2002) have shown that human and rabbit antibodies to the NC16A portion of BPAg2 induced neutrophil-dependent subepidermal splits in cryo-sections of human skin. This *in vitro* model is dependent on binding of antibody to the extracellular portion of BPAg2. There is little question that neutrophils in combination with BMZ antibodies will induce BMZ separation. Our *in vivo*

model is not dependent on IgG antibodies to BPAg2 and does not produce neutrophil accumulation at the BMZ. Although neutrophil accumulation at the BMZ is not a characteristic of human BP, the presence of neutrophil elastase in human BP lesions and the *in vitro* effect of neutrophils indicate that neutrophils may have a role in our model. It is likely that some neutrophil infiltration has occurred in our model, although we have detected only rare neutrophils with routine staining. There may well be neutrophil elastase present in the tissue and blister fluid in our model. We plan subsequent experiments to evaluate the role of neutrophils, including depletion of neutrophils from the test animals. We have not shown that eosinophils are required for the inflammatory response in this model, only that they are present. Eosinophils may be bystanders in the inflammatory response or they may be essential. We plan experiments that deplete mouse eosinophils in this model in order to experimentally address this issue. However, for the purpose of this initial report, it is important to realize that our model produces two histological hallmarks of human BP, eosinophil infiltration and a lamina lucida blister. Any possible role of the eosinophil in producing the lamina lucida blister remains to be determined.

Liu *et al.* (1993) developed a mouse model used to study BP. It utilizes the passive transfer of IgG fraction rabbit antibody directed against the mouse NC16A domain of BP180. A visible BMZ blister as well as IgG and complement deposition at the BMZ develop within 12–24 hours. These are classical findings in human BP. However, this system utilizes rabbit antibody, which is known to avidly fix complement. The lack of eosinophils in the infiltrate suggests that an immunopathological process is being produced, which is different from that of human BP. It may be that a classical Arthus reaction has been produced to a tissue antigen by a complement-fixing antibody.

The model we have developed offers advantages for the study of BMZ autoimmune bullous disease. It allows the evaluation of the pathogenic role of individual antibody classes. The current work demonstrates the value of that approach. We have found that multiple immunoglobulins are frequently present in the skin of BP patients (Zone *et al.*, 1994). This model also has the potential to allow the study of the effect of IgE in combination with other immunoglobulins.

MATERIALS AND METHODS

Animals

Male C.B-17/lcr-SCID mice 5–6 weeks of age were obtained from Charles River (Wilmington, MA). Animals were housed for 2–4 weeks before grafting, allowing them to acclimate to the new environment in our housing facility. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the University of Utah. Preliminary experiments indicated that the previously utilized athymic mouse model resulted in the production of mouse antibodies against the cutaneous BMZ on direct immunofluorescence in all areas of the biopsies studied in approximately 20% of mice. Consequently, we have utilized SCID mice and grafted human foreskin to the mice as previously described (Zone *et al.*, 2004). Immunopathological evaluation of the graft showed no evidence of a

humoral immune response to the human skin graft. Neonatal foreskins from elective circumcision were obtained and transplanted onto the upper backs of the mice after being trimmed of excess fat. Skin was sutured, dressed, and allowed to heal for 10–14 days before the dressings were removed. Grafts were allowed to mature for 8–10 weeks and only those that showed no signs of inflammation or rejection were used.

Hybridoma cell line generation

IgG and IgA hybridomas were produced as described previously (Zone *et al.*, 2004). Acridine orange ICR191 (Sigma, St Louis, MO) stimulation of our previously described IgG1 anti-LABD97 hybridoma led to the recovery of an IgE isotype switch variant (Paizi *et al.*, 1995; Zone *et al.*, 2004). In brief, the IgG hybridoma was treated with ICR191 in cell culture and a subsequent IgE isotype switch variant was isolated by decreasing cell density plating. Positive producers were identified by enzyme-linked immunosorbent assay spot assay on plates seeded with goat anti-mouse IgE antibody (Bethyl Laboratories, Montgomery, TX) (Spira *et al.*, 1996). Immunoblot of the IgE hybridoma antibody demonstrated reactivity with the LABD97 antigen (a fragment of BPAg2) but not intact BP180 (not shown). This is identical to the pattern seen for the parent IgG1 anti-LABD97 hybridoma. Indirect immunofluorescence on a human split skin substrate was performed using the supernatant from the IgE hybridoma. There was strong binding of mouse IgE BMZ antibody to the epidermal side of the BMZ (results not shown). This was identical to the pattern seen previously for the parent IgG1 antibody (Zone *et al.*, 2004). Indirect immunofluorescence was performed on mouse and human skin with the hybridoma-derived IgE. This demonstrated strong binding to human BMZ and no binding to mouse BMZ. This implies that the epitope recognized by our hybridoma antibody is present in human but not in mouse BMZ.

Hybridoma cell line growth medium consisted of RPMI-1640 containing 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and fungicide. Two control hybridomas, one producing phenylarsonate-specific mouse IgE monoclonal antibody (SE1.3) and the other TNP-specific mouse IgE monoclonal antibody (IGELB4), were purchased from American Type Culture Collection (Manassas, VA).

Backpack tumor production

Backpack tumors were produced as described previously (Winner *et al.*, 1991; Haneberg *et al.*, 1997). Briefly, for each mouse, $1-2 \times 10^6$ hybridoma cells were washed twice in phosphate-buffered saline, suspended in 0.2 ml phosphate-buffered saline, and then injected subcutaneously into the upper back of the grafted SCID mice. To ensure a maximum number of cells in log phase, a medium change was always performed 6 hours before injection. Tumors were visible at the injection site after 11–14 days. Tumor size for all mice selected in the study ranged from 2 to 3 cm in diameter when the experiment was terminated on day 21. The subcutaneous backpack tumor was located in a site as far as possible from the human skin graft. In no animals were the backpack tumors closer than 3–4 cm to the graft.

Intradermal injection of hybridoma supernatant into the human skin graft

To further evaluate that the IgE LABD97 antibody itself was responsible for the inflammatory response, we prepared IgE from

ascites fluid of mice bearing intraperitoneal IgE LABD97 and IgE TNP hybridomas. Preparations consisting of 100 ng of IgE in 50 µl of phosphate-buffered saline were used. This concentration was found to be optimal in preliminary experiments. These preparations were injected intradermally into human skin grafts of SCID mice bearing no subcutaneous hybridoma. The mice were observed for 1 hour for erythema and biopsies were performed at 24 hours.

Animal and graft evaluation

Graft and mouse skin from the test and control groups was visually examined during the course of the experiment (18–21 days) for erythema. At days 18–21 on visual examination, the mice were noted to be actively scratching themselves. They were not able to reach the graft itself, but were scratching the areas on their body that they were capable of reaching. Biopsies were taken at selected time points and immediately frozen or embedded in paraffin. These time points consisted of day 0 and days 8–11. The graft was removed in its entirety when the animals were killed at days 18–21 (end of experiment). Five biopsies taken at days 8–11 when visible erythema developed were evaluated for mast cell degranulation. Intradermal injection of goat anti-mouse IgE (5 µg/50 µl, Bethyl Labs) into the graft on the control mice was performed on day 21 (Gronneberg and Strandberg, 1985). At the conclusion the animals were killed, serum was collected, and mouse skin and the human graft were excised. Cryosections were prepared and stained by direct immunofluorescence for the presence of mouse antibodies and mast cells. Fluorescein isothiocyanate conjugated goat anti-mouse IgG (Vector, Burlingame, CA), goat anti-mouse IgM and C3 (Cappel, Irvine, CA), goat anti-mouse IgA (Sigma, St Louis, MO), rat monoclonal anti-mouse IgE (Becton Dickinson, San Diego, CA), and rhodamine conjugated avidin (Vector) for the detection of human and murine mast cells, and mouse monoclonal anti-human mast cell tryptase (Promega, Madison, WI) were stained by conventional staining procedures. Paraffin-embedded sections were prepared and stained with hematoxylin and eosin and toluidine blue. Mouse eosinophil major basic protein was stained with antibody supplied by Dr Kristin Leiferman.

Measurement of mouse IgE antibodies

IgE levels in mouse serum were determined by a mouse IgE quantitative ELISA (Bethyl Laboratories, Montgomery, TX).

Immunomapping of blisters

Several graft biopsies from the test mouse group showing blisters at the BMZ were stained by indirect immunofluorescence to determine the location of the separation. Cryosections 6 µm thick were stained with a mAb specific for human collagen IV (BD Biosciences, San Diego, CA) and human serum reactive with BPAg2 antigen (Meyer *et al.*, 1990).

Quantification of mast cell degranulation

The extent of mast cell degranulation was determined by dividing the difference of the average number of mast cells seen at the time of visible erythema (8–10 days) by the average seen at 0 hour. Five dermal papillary fields (original magnification $\times 400$) were considered in each group. This number is expressed as a percentage. Mast cells were identified by fluorescent microscopy with rhodamine-labeled avidin. Mouse skin as well as human graft was examined from both the test animals and controls.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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