Evidence that Anti-Type VII Collagen Antibodies Are Pathogenic and Responsible for the Clinical, Histological, and Immunological Features of Epidermolysis Bullosa Acquisita

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Epidermolysis bullosa acquisita (EBA) is an autoimmune blistering disease characterized by autoantibodies to type VII (anchoring fibril) collagen. Therefore, it is a prototypic autoimmune disease defined by a well-known autoantigen and autoantibody. In this study, we injected hairless immune competent mice with purified immunoglobulin G (IgG) fraction of serum from rabbits immunized with the non-collagenous amino-terminal domain (NC1) of human type VII collagen, the domain known to contain immunodominant epitopes. As a control, identical mice were injected with the IgG fraction of serum from non-immunized rabbits. Mice injected with immune IgG developed subepidermal skin blisters and erosions, IgG deposits at the epidermal–dermal junction of their skin, and circulating anti-NC1 antibodies in their serum-all features reminiscent of patients with EBA. Similar concentrations of control IgG purified from normal rabbits did not induce disease in the mice. These findings strongly suggest that autoantibodies that recognize human type VII collagen in EBA are pathogenic. This murine model, with features similar to the clinical, histological, and immunological features of EBA, will be useful for the fine dissection of immunopathogenic mechanisms in EBA and for the development of new therapeutic interventions.

Key words: animal model/autoimmunity/basement membrane/bullous disease/skin/type VII collagen
J Invest Dermatol 124:958–964, 2005

Epidermolysis bullosa acquisita (EBA) is an incurable autoimmune blistering disease of the skin characterized by skin fragility, blisters in trauma-prone sites, and scarring with milia formation and nail dystrophy (Roenigk *et al*, 1971). EBA is a prototypic autoimmune disease in which EBA patients have autoimmune immunoglobulin G (IgG) antibodies in their blood and skin that are directed against a specific structure in the skin called anchoring fibrils (Woodley *et al*, 1984, 1986, 1988). These structures are responsible for holding together the two main layers of skin: the epidermis and dermis (Briggaman and Wheeler, 1975). Anchoring fibrils are located within the basement membrane zone (BMZ) between the epidermis and dermis. They are composed of type VII collagen (Sakai *et al*, 1986; Keene *et al*, 1987; Burgeson, 1993). EBA autoantibodies bind to type VII collagen within anchoring fibrils and this binding is associated with a diminution of normal anchoring fibrils in the patient's skin and subsequent epidermal–dermal disadherance.

Type VII collagen is composed of three identical α chains (Sakai *et al*, 1986; Keene *et al*, 1987; Burgeson, 1993). Each α chain consists of a central collagenous domain flanked by a 145 kDa non-collagenous amino-terminal domain (NC1) and a 30 kDa carboxyl-terminal domain (Lunstrum *et al*, 1986, 1987). The primary binding site within the type VII collagen molecule for EBA autoantibodies is the NC1 domain (Gammon *et al*, 1993; Lapiere *et al*, 1993; Jones *et al*, 1995).

The "Koch's postulate" of autoimmune diseases shows that the disease can be duplicated in an animal by injecting into the animal an antibody specifically directed against the autoantigen self-protein (in this case type VII collagen—so called "passive transfer" of the disease) (Witebsky, 1966; Rose and Bona, 1993). The duplication of the disease in the animal proves that the antibody is "pathogenic" and responsible for the disease phenotype. This has been accomplished in the autoimmune diseases pemphigus vulgaris, pemphigus foliaceus, and bullous pemphigoid (Roscoe *et al*, 1985; Anhalt *et al*, 1993; Liu *et al*, 1993).

Several independent lines of evidence derived from clinical, histologic, and immunologic studies have implicated autoimmunity element in the pathogenesis of EBA. Consistent induction of blisters in an animal by the passive transfer of EBA IgG autoantibodies into the animal, however, has not been achieved, despite numerous attempts (Shigemoto *et al*, 1988; Chen *et al*, 1992; Borradori *et al*, 1995). In earlier studies, when IgG autoantibodies were injected into neonatal mice, they bound to the animal's anchoring fibrils, fixed complement, and generated an inflammatory infiltrate at the dermal–epidermal junction (DEJ), but

Abbreviations: BMZ, basement membrane zone; DEJ, dermal epidermal junction; DIF, direct immunofluorescence; EBA, epidermolysis bullosa acquisite; IgG, immunoglobulin G; IIF, indirect immunofluorescence; NC1, 145 kDa non-collagenous domain of type VII collagen

no dermal–epidermal separation was observed (Shigemoto *et al*, 1988; Borradori *et al*, 1995). Likewise, Chen *et al* (1992) also injected EBA serum and whole human blood into SCID mice transplanted with human skin grafts but failed to induce blisters in either murine or grafted human skin. Nevertheless, the EBA serum IgG infused into the animal bound to the BMZ of both the murine skin and the transplanted human skin (Chen *et al*, 1992).

In this study, we raised a high titer antibody to recombinant human type VII collagen, specifically, the highly antigenic NC1 domain (Chen *et al*, 1997). The resulting rabbit antibody recognized both human and mouse type VII collagen. We purified IgG fractions of the rabbit immune serum and injected them intradermally into immunocompetent, hairless mice. We found that passively transferring anti-NC1 antibodies into mice consistently induced a subepidermal blistering disease resembling the clinical, histological, and immunological features of human EBA.

Results

Preparation and purification of recombinant human NC1 We have previously expressed large quantities of the recombinant NC1 domain of human type VII collagen in human epithelial 293 cells (Chen *et al*, 1997). As shown in Fig 1*A*, the 293 cells cannot constitutively synthesize and secrete human type VII collagen (*panel a*, *lane 1*). After the cells were transfected with the pRC/CMV vector containing human cDNA for the NC1 domain of type VII collagen, the cells synthesized and secreted the 145 kDa NC1 domain of type VII collagen (*lane 2*). We purified the recombinant human NC1 protein from conditioned media to homogeneity by column chromatography as previously described (*panel b*) (Chen *et al*, 1997). The recombinant NC1 was then used to immunize rabbits to produce polyclonal anti-NC1 antibodies.

The IgG fraction of immune serum is specific for NC1 We purified IgG from immunized rabbit sera. The antigenic specificity of the immune IgG was characterized by immunoblot analysis as shown in Fig 1*B*. The anti-NC1 IgG only labeled the 145 kDa NC1 (*lane 1*) and did not label other matrix proteins including type I collagen, type IV collagen, fibronectin, laminin-1, and laminin-5 (Fig 1*B*, *lanes 2–6*).

By indirect immunofluorescence (IIF) against human and murine skin (Fig 1*C*), the purified IgG fraction from the rabbit anti-NC1 sera strongly labeled the BMZ of both human and mouse skin, whereas the IgG fraction from control rabbit sera did not. IIF testing against salt-split human skin substrate showed that the antibody labeled the dermal side, consistent with labeling type VII (anchoring fibril) collagen (Gammon *et al*, 1984, 1990). Serial dilutions of the IgG fraction showed that the IgG contained anti-type VII collagen antibodies against human and murine skin at titers over 1:100,000 and against salt-split human skin at greater than 1:200,000. Further, the IIF staining of human skin substrate could be completely blocked by pre-absorption of the IgG fraction with NC1-affinity column (*panel D*) but not by pre-absorption with type IV collagen, fibronectin, and laminin-1 (data not shown).

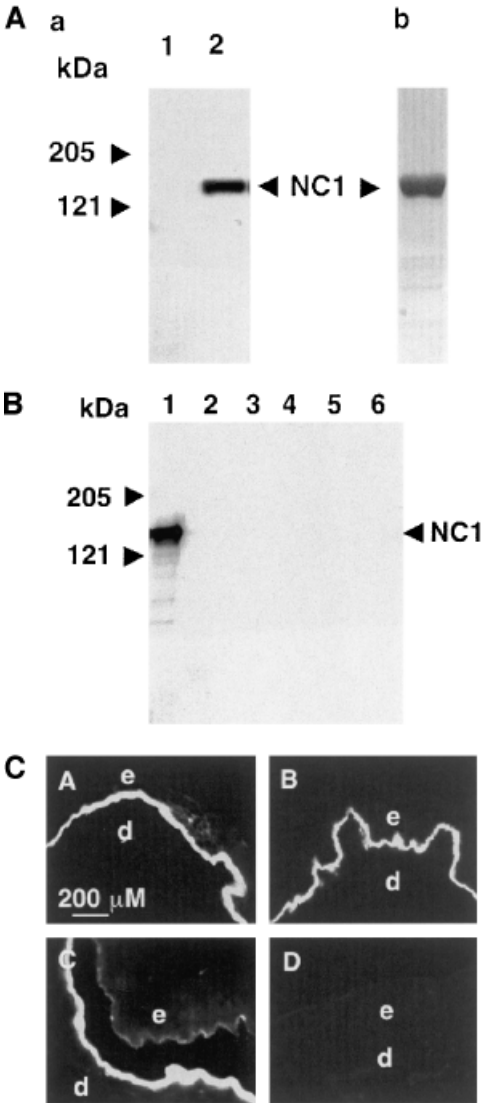

Figure 1
Production and characterization of purified rabbit anti-NC1 (145 kDa non-collagenous domain of type VII collagen) antibodies. (*A*) Expression and purification of recombinant NC1 domain of human type VII collagen from stably transfected 293 cells. (*a*) Conditioned media from parental 293 cells (*lane 1*) and 293 cells stably transfected with an expression vector coding for human NC1 (*lane 2*) were concentrated and subjected to 6% SDS-PAGE followed by immunoblot analysis using a monoclonal antibody to NC1. The positions of the 145 kDa NC1 and molecular weight markers are indicated. (*b*) Six percent SDS-PAGE and Coomassie blue staining of purified 145 kDa NC1. (*B*) Specificity of rabbit anti-NC1 antibody for NC1. Purified recombinant NC1 as well as other extracellular matrix components (400 ng per well) were separated on 6% SDS-PAGE and transferred to nitrocellulose membranes before incubation with rabbit anti-NC1 antibody at a dilution of 1:5000 and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) (1:5000) followed by ECL detection. Lanes 1–6 are NC1, type I collagen, type IV collagen, fibronectin, laminin-1, and laminin-5, respectively. The location of the 145 kDa of recombinant NC1 and molecular weight markers are indicated. (*C*) Immunolabeling of mouse and human skin with purified rabbit anti-NC1 IgG. Immunofluorescence staining was performed on human skin (*panels A* and *D*), mouse skin (*panel B*), and salt-split human skin (*panel C*). The tissue was labeled with a purified rabbit anti-NC1 antibody (*panels A–C*) and a flowthrough IgG fraction from an NC1-affinity column (*panel D*) at a dilution of 1:5000 and a fluorescein isothiocyanate-conjugated goat-anti rabbit IgG. Note that the rabbit anti-NC1 antibody strongly labeled the basement membrane zone (BMZ) of both mouse and human skin and the dermal floor of salt-split human skin. In contrast, IgG depletion of reactivity to the NC1 domain (flowthrough fraction) did not label the BMZ. d, dermis; e, epidermis.

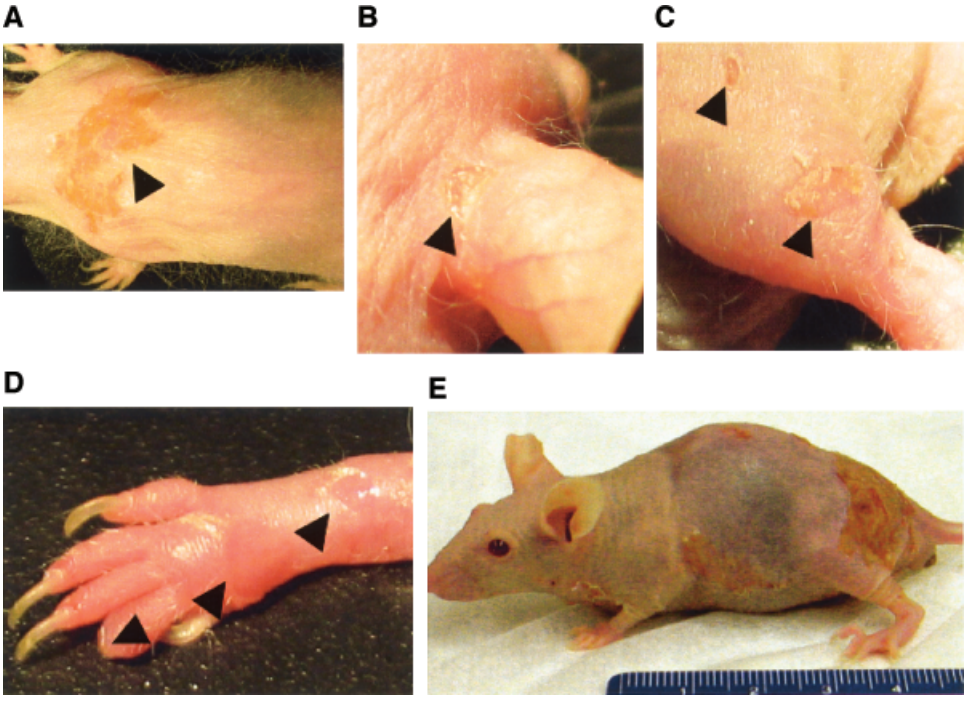

Figure 2
Cutaneous lesions induced in SKH1 mice by injection of rabbit anti-NC1 (145 kDa non-collagenous domain of type VII collagen) immunoglobulin G (IgG). SKH1 mice were injected intradermally with rabbit anti-NC1 IgG at 1 mg per g body weight once every day for 6 consecutive days (*panels A–D*) or 10 consecutive days (*panel E*). Four days after the injections were started (4 ×), animals developed numerous blisters, erosions, and crusts forming from ruptured blisters at the site of injection (*A*). These lesions were observed on the animals' ears (*B*) and legs (*C*) at 8 d and paws (*D*) at 24 d after initial injections (6 ×). Nail loss was also observed in some mice (*D*). Mice continued to develop new, increasingly severe, and widespread erosions even 1 mo (*E*) after the initial series of antibody injections (10 ×).

IgG fractions from immunized rabbits induced skin fragility, blisters, and erosions in mice SKH1 mice were given daily intradermal injections of purified IgG fractions prepared from the rabbit anti-NC1 antisera and control rabbit sera at doses from 0.1 to 1 mg per g body weight. As shown in Fig 2, animals injected with the rabbit anti-NC1 IgG (n = 15) showed numerous erosions and crusts forming from ruptured blisters as early as 4 d after the initial injection at the injection site (*panel A*). With time, additional erosions were observed, particularly on the ears at 8 d (*panel B*), hips at 8 d (*panel C*), and paws at 24 d (*panel D*)—trauma-prone sites where the animals scratch themselves. All of the 15 mice developed blisters and then erosions at the injection site, and most, but not all mice, developed bullae and erosions on the ears, hips, and paws. Nail loss was also observed in 80% of injected mice (*panel D*). Mice continued to develop new, increasingly severe, widespread erosions even 1 mo after the initial series of 10 antibody injections (*panel E*). We observed cutaneous lesions induced by the rabbit anti-NC1 in all the injected mice (n = 15). In contrast, no blisters or erosions developed in the mice injected with rabbit control IgG (n = 5). We did not formally measure the percentage involvement of the animals' body surface area with bullae and erosions and correlate this with the doses of immune IgG given. Nevertheless, from the photographic documentation of the mice, it is our overall impression that a greater percentage of the total body surface area was involved with erosions and blisters when the mice were given higher (> 1 mg per g body weight) doses of rabbit anti-NCI IgG. In fact, animals given doses less than 0.2 mg per g body weight of immune IgG did not develop any skin lesions. Similarly, nail loss only occurred in mice given higher IgG doses (≥ 1 mg per g body weight—12 of 12 mice).

Histological and immunological examination of mice injected with immune IgG Histological examination of mouse skin injected intradermally with rabbit anti-NC1 IgG demonstrated a full sub-epidermal blister with a clean separation between the epidermis and dermis (Fig 3*A*). There was also a scattered dermal inflammatory infiltrate. In contrast, mice injected with similar concentrations of rabbit control IgG had no skin pathology (Fig 3*B*). Direct immunofluorescence (DIF) of perilesional and lesional skin of all mice injected with rabbit anti-NC1 IgG showed strong deposition of tissue-bound, rabbit, anti-NC1 IgG at the DEJ between the epidermis and dermis of the mouse skin (Fig 4). Also, in many mice, murine complement was detected at the DEJ (Fig 4). Within the dermal inflammatory infiltrate, neutrophils could be detected in small numbers (Fig 4). There were no IgG or C3 deposits observed at the BMZ of mice injected with rabbit control IgG. Further, neutrophils were not detected in the skin of mice injected with rabbit control IgG.

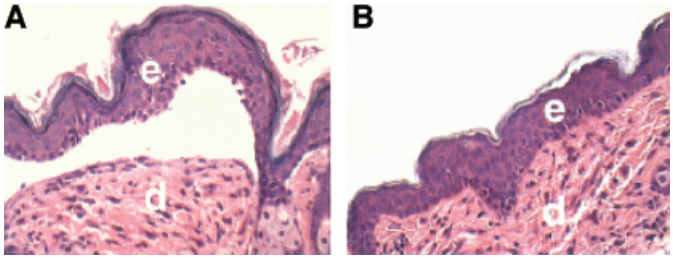

Figure 3
Histological examination of lesional skin of SKH mice injected with rabbit anti-NC1 (145 kDa non-collagenous domain of type VII collagen) immunoglobulin G (IgG). (*A*) Hematoxylin and eosin staining of lesional murine skin revealed separation of the epidermis (*e*) from the dermis (*d*). This histologic finding is similar to that seen in lesional skin of epidermolysis bullosa acquisita patients. No epidermal–dermal separation was seen in mice receiving equivalent amounts of rabbit control IgG (*B*).

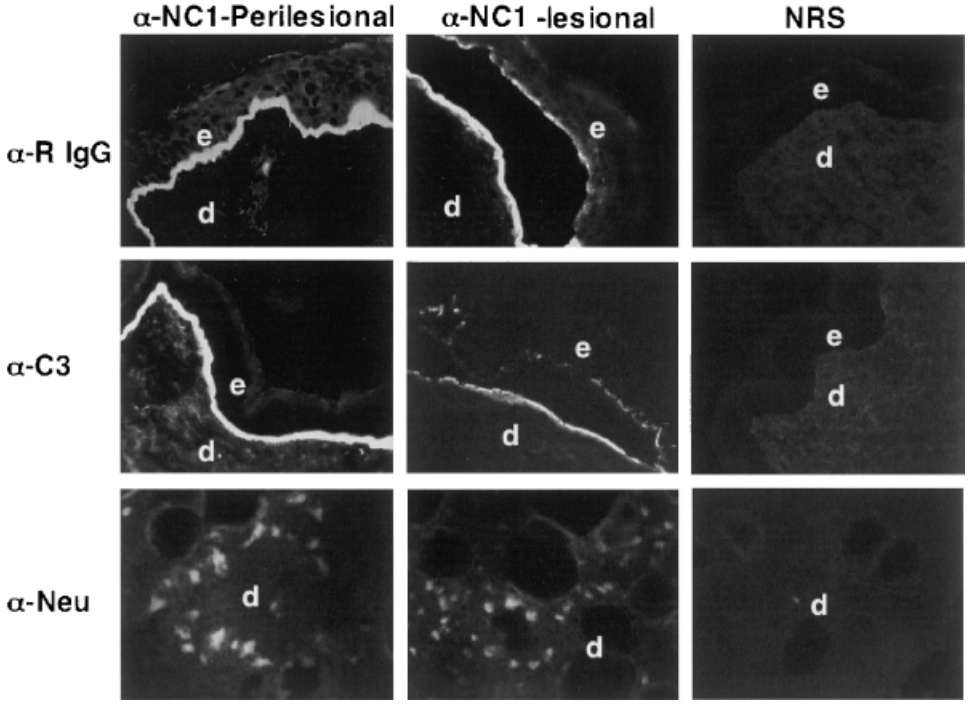

Figure 4
Immunofluorescence analysis of SKH1 mice skin injected with rabbit anti-NC1 (145 kDa non-collagenous domain of type VII collagen) immunoglobulin G (IgG). Cryosections of perilesional and lesional skin were labeled with fluorescein isothiocyanate (FITC) goat anti-rabbit IgG (α-R IgG), FITC-goat anti-mouse C3 (α-C3), and FITC-goat anti-mouse neutrophils (α-Neu), respectively. Note that the linear deposits of rabbit IgG and murine C3 were found at the basement membrane zone of perilesional and lesional mouse skin injected with rabbit anti-NC1 IgG. Please also note the scattered neutrophilis in the dermis. In contrast, in mice receiving equivalent amounts of purified rabbit control IgG (NRS), no deposits of rabbit IgG or murine C3 were detected.

Evaluation of sera from mice injected with rabbit IgG Mice injected with rabbit anti-NC1 IgG had high titers of circulating antibody (1:20,000–1:50,000) when assayed by IIF against either normal or salt-split human skin. As shown in Fig 5, serum from the diseased mice had rabbit antibodies that bound to the DEJ of normal human skin (*panel A*) and the dermal side of the salt-split human skin (*panel B*), reminiscent of EBA patient sera. In contrast, serum from mice injected with control IgG showed no binding to the DEJ of human skin (*panel C*) even at dilutions of 1:10.

Discussion

In this study, we have shown that the sub-epidermal, autoimmune bullous disease, EBA, can be passively transferred into hairless, immune competent mice using rabbit anti-NC1 IgG. We have shown previously that the NC1 domain of type VII collagen is a highly antigenic part of the type VII collagen α chain and that the sera of most patients with EBA contain IgG autoantibodies against NC1 (Lapiere *et al*, 1993). The mice injected with anti-NC1 IgG from immunized rabbits developed sub-epidermal blisters and erosions, reminiscent of the skin lesions seen in EBA patients. Like many EBA patients, the mice often had loss of nails on their paws. Also, like EBA patients, these mice exhibited IgG deposits at the DEJ by DIF, one of the critical diagnostic tests for human EBA. Like many EBA patients, the mice also had anti-NC1 IgG antibodies circulating in their blood as demonstrated by salt-split IIF. Taken together, the mice injected with immune IgG exhibited clinical, histological, and immunological features akin to patients with EBA.

Several independent lines of evidence derived from clinical, histological, and immunological studies have implicated autoimmunity in the pathogenesis of EBA. Evidence for the pathogenic role of EBA auto-antibodies also comes from the observation that when patients with systematic lupus erythematosus (SLE) develop autoantibodies to the EBA antigen, they develop skin blisters and fall into a subset called "bullous SLE" (Gammon *et al*, 1985). Patients with SLE have an enhanced immune system and frequently make autoantibodies to a variety of tissues. Normally, SLE patients do not have skin fragility or blisters. But when SLE patients serendipitously make autoantibodies to type VII collagen, a widespread blistering eruption of the skin ensues. This "experiment of nature" suggests that EBA autoantibodies are pathogenic and capable of inducing disadherence between the epidermis and dermis.

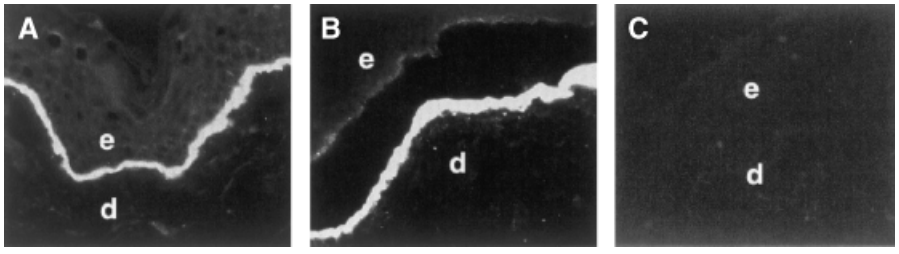

Figure 5
Immunolabeling of human skin with serum from mice injected with rabbit anti-NC1 (145 kDa non-collagenous domain of type VII collagen) immunoglobulin G (IgG). Sections of normal human skin (*A*, *C*) and salt-split human skin (*B*) were stained with mouse serum obtained from mice injected with either rabbit anti-NC1 IgG (*A*, *B*) or control IgG (*C*) at a dilution of 1:2000. Note that circulating antibodies labeling the basement membrane zone of human skin and the dermal floor of salt-split human skin were found in the serum samples from mice injected with anti-NC1 IgG but not those injected with control IgG.

Our laboratory and others previously have attempted to transfer EBA passively into mice using concentrated IgG serum fractions and even whole blood from EBA patients without success (Shigemoto *et al*, 1988; Chen *et al*, 1992; Borradori *et al*, 1995). When IgG autoantibodies were injected into neonatal mice, they bound to the animal's anchoring fibrils, fixed complement, and generated an inflammatory infiltrate in the dermis, but no dermal–epidermal separation occurred.

With this novel EBA animal model being developed, several possibilities could explain previous failures. First, in the study by Borradori *et al* (1995), the investigators used neonatal mice for IgG passive transfer with only two injections and a limited incubation time. The turnover of anchoring fibrils is thought to be low (Burgeson, 1993). Existing resident anchoring fibrils in the animals may have continued to function despite the presence of EBA autoantibodies. It is possible that multiple injections of EBA autoantibodies with a longer time period may be needed to reproduce the manifestations of EBA in an animal model. In this study, we injected adult mice every day for up to 10 d.

Another potential factor may be the amount of anti-type VII collagen antibody injected. In the previous studies, we injected serum IgG fractions from EBA patients known to have IgG autoantibodies to type VII collagen. Most EBA patients have low titers of anti-type VII collagen antibodies and much lower levels of NC1-specific IgG in their serum. The amount of actual anti-type VII collagen antibodies in the injected serum IgG fractions was likely extremely low. In the study by Shigemoto *et al* (1988), EBA passive transfer was attempted in adult mice for 3 consecutive days, but the amount of IgG fraction injected was very low, only 0.13 mg per g body weight. In this study, we raised rabbit antibodies to the NC1 domain of type VII collagen. The titers in the IgG fraction purified from the immunized animals were extremely high ($>$1:100,000 against human and mouse skin sections by IIF and over 1:200,000 on human salt-split skin substrate). Moreover, since the pathogenic epitopes on type VII collagen are concentrated on the NC1 domain, we likely created anti-type VII collagen antibodies directed against the pathogenic epitopes. In addition, we administered multiple injections using IgG fraction at doses of 0.3–1.0 mg per g body weight.

The NC1 domain of type VII collagen constitutes the major immunodominant epitopes that are targeted by the majority of EBA sera (Gammon *et al*, 1993; Lapiere *et al*, 1993; Jones *et al*, 1995). The fact that the IIF staining of human skin substrate could be completely abolished by pre-absorption of the anti-NC1 rabbit IgG fraction with NC1, but not with type IV collagen, fibronectin, and laminin-1, demonstrates the specificity of the anti-NC1 antibodies that bind to the BMZ of mouse and human skin.

EBA patients often develop blisters and erosions in oral mucosa. In these studies, we did not examine formally whether the experimental mice had significant involvement of the oral mucosa. The immune IgG-injected mice, however, did not exhibit significant weight loss even though they had many months of active skin lesions. This suggests that the animals did not have significant oral mucosal involvement that inhibited their ability to eat and drink.

Anchoring fibrils are reduced in the skin of EBA patients, but the underlying mechanism leading to this reduction is unknown (Nieboer *et al*, 1980; Yaoita *et al*, 1981). Because EBA often occurs with minimal clinical or histological inflammation, it has been hypothesized that defective epidermal–dermal adherence in EBA involves autoantibodies targeting functional epitopes within the type VII collagen molecule. This could perturb critical direct interactions between type VII collagen and other extracellular components within the DEJ such as type IV collagen, laminin-5, and fibronectin (Lapiere *et al*, 1994; Chen *et al*, 1997; Chen *et al*, 1999). We and others have shown that specific domains within the NC1 domain of the type VII collagen α chain have affinity for laminin-5, type IV collagen, and fibronectin. These interactions may be necessary for keeping the DEJ intact (Lapiere *et al*, 1994; Chen *et al*, 1997; Chen *et al*, 1999). By this mechanism, it is possible to envision how a patient could have skin fragility and poor adherence between the epidermis and dermis in association with a few normal anchoring fibrils and also have minimal inflammation in the lesions.

Classical EBA is associated with skin fragility and subepidermal blisters with minimal inflammation (Woodley *et al*, 1984). There is also an inflammatory variety of EBA characterized by vesiculobullous lesions on inflamed skin. The mice that received immune IgG clearly exhibited skin fragility and nail loss, features of a mechanobullous disease. Nevertheless, there was some mild inflammation with the presence of a few neutrophils in the dermis.

In the EBA murine model, the administered immune IgG fixed murine complement at the DEJ, which is a feature of sera from both classical EBA and inflammatory EBA (Mooney and Gammon, 1990). Interestingly, EBA antibodies are more potent activators of complement than are antibodies in the sera of patients with bullous pemphigoid, a prototypic inflammatory autoimmune bullous disease (Mooney *et al*, 1992). The presence of complement-activating IgG autoantibodies does not correlate with the inflammatory or noninflammatory EBA clinical phenotypes (Gandi *et al*, 2000).

In summary, this study provides the first evidence that antibodies directed against the NC1 domain of human type VII collagen are pathogenic when passively transferred into hairless immunocompetent mice. This experimental mouse model should be useful for dissecting the molecular and immunological mechanisms of subepidermal blister formation in EBA and for developing more effective therapy for EBA.

Materials and Methods

Mice SKH1 mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and hosted at the University of Southern California Facility. These are hairless mice with an intact immune system. The animals were injected with purified rabbit anti-NC1 IgG or non-immune rabbit control IgG between the ages of 6 and 12 wk. All animal studies were conducted using protocols approved by the University of Southern California Institutional Animal Use Committee.

Generation of rabbit anti-type VII collagen antibody The NC1 was generated in human 293 cells by stable transfection with a pRC/CMV expression vector, and the recombinant NC1 was

purified to homogeneity from conditioned media, as previously described (Chen *et al*, 1997). New Zealand white rabbits were immunized with 10 mg of the recombinant protein injected intradermally at time 0, 2 wk later, and then with 5 mg every 4 wk. Retro-occular venous blood was sampled from the animals and tested by IIF against normal human skin substrate as described (Gammon *et al*, 1984, 1985, 1990). Sera from multiple bleedings of the animal were pooled, and the IgG fractions were purified using protein G Sepharose Fast Flow chromatography following the manufacture's recommendation (Amersham Biosciences, Uppsala, Sweden). IgG fractions were dialyzed against phosphate-buffered saline (PBS), concentrated by Centricon Plus-20 ultrafiltration (Amicon, Lexinton, Massachusetts) to 100 mg per mL, filtered sterilized, and stored at -20°C. We then retested it by IIF (antibody dilutions ranged from 1:500 to 1:200,000) on salt-split skin substrate, western blot analyses, and ELISA as described (Gammon *et al*, 1984, 1990; Woodley, 1990; Chen *et al*, 1997). Identical procedures were performed using IgG fractions purified from non-immune normal rabbit serum.

Administration of anti-NC1 IgG to experimental animals SKHI mice were injected intradermally with rabbit anti-NC1 ($n = 15$) or rabbit control IgG ($n = 5$) once every day for up to 10 d and observed every day. IgG doses ranged from 0.3 to 1 mg per g body weight. The animals were photographed at each observation. Skin erythema, blisters, and erosions were recorded. Mice that developed blisters had skin biopsies from the blisters and non-blistered normal-appearing skin within 0.5 cm of a blister. Histological sections of lesional and perilesional skin were fixed in 10% buffered formalin and stained with hematoxylin and eosin. Both lesional and non-lesional tissues were subjected to DIF staining as previously described. Monospecific fluorescein isothiocyanate (FITC)-conjugated sera were obtained commercially: goat anti-rabbit IgG (Sigma, St Louis, Missouri), monospecific goat anti-mouse C3 (Cappel Laboratories, Durham, North Carolina), and FITC-conjugated goat anti-mouse neutrophils (Cedarlane, Ontario, Canada). The Cedarlane monoclonal antibody to murine neutrophils is commercially available and has been used in other murine studies (Hirsch and Gordon, 1983). Nevertheless, although Cedarlane tested a number of murine strains with this antibody, we could not find in the literature a reference in which SKH1 mice were specifically tested. Therefore, in order to be sure that the antibody specifically stained neutrophils in SKH1 mice, we made thick blood smears from retro-orbital blood samples of two SKH1 mice and labeled the smears with the antibody diluted 1:50 in PBS. The Cederlane antibody labeled neutrophils in the smear (data not shown). Photographs of immunolabeled tissues were obtained with a Zeiss Axioplan fluorescence microscope equipped with a Zeiss Axiocam MRM digital camera system (Carl Zeiss, Thornwood, New York).

Sera were obtained from mice at the time of biopsy and examined for anti-type VII collagen antibodies in the murine serum by IIF, western blot analysis, and ELISA, as described above and previously published (Gammon *et al*, 1984, 1990; Woodley, 1990; Chen *et al*, 1997). Animals given rabbit control IgG were studied in an identical fashion.

This work was presented in part at the annual national meeting of the Society of Investigative Dermatology at Los Angeles, May 2002. This work was supported by grants RO1 AR47981 (M. C.), RO1 AR33625 (D. T. W.), and RO1 AI40768 (L. Z.) from the National Institutes of Health.

DOI: 10.1111/j.0022-202X.2005.23702.x

Manuscript received August 20, 2004; revised December 29, 2004; accepted for publication January 3, 2005

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