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.

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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).

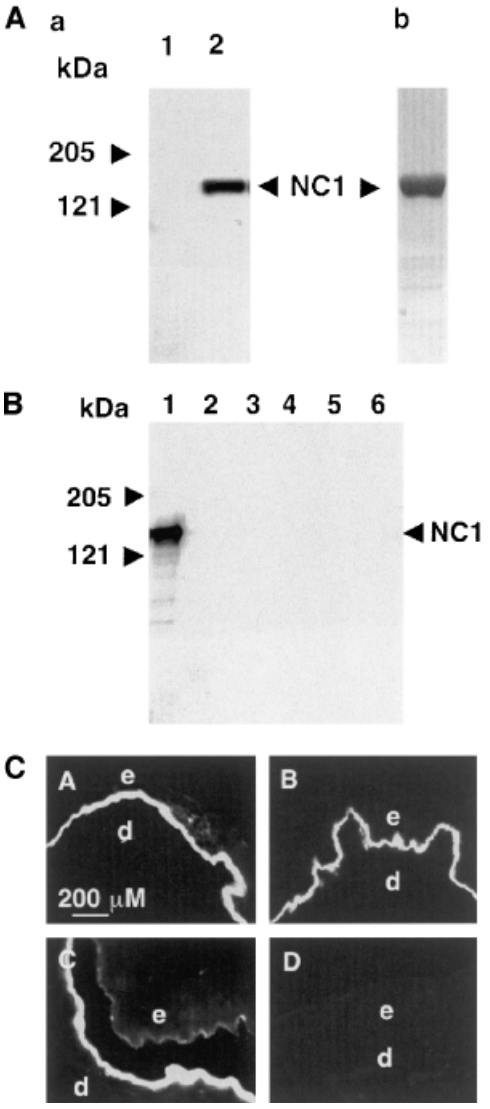

Figure 1
Production and characterization of purified rabbit anti-NC1 (145 kDa non-collagenous domain of type VII collagen) antibodies. (*A*) Expression and purification of recombinant NC1 domain of human type VII collagen from stably transfected 293 cells. (*a*) Conditioned media from parental 293 cells (*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.