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für Haut- und Geschlechtskrankheiten
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Untersuchungen zur Pathogenität von Autoantikörpern gegen Typ VII Kollagen von Patienten mit Epidermolysis bullosa acquisita

Pathogenicity of autoantibodies to type VII collagen from patients with epidermolysis bullosa acquisita

INAUGURAL-DISSERTATION

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Abbreviations

AEBSF – aminoethylbenzene sulfonylfluoride

BMZ – basement membrane zone

BP – bullous pemphigoid

BSA – bovine serum albumin

DEJ – dermal-epidermal junction

EBA – epidermolysis bullosa acquisita

EDTA – ethylenediamine tetraacetic acid

IC – immune complexes

IF – immunofluorescence

LAD – linear IgA disease

LAD-1 – linear IgA disease antigen 1

MoAb – monoclonal antibody

NBT – nitroblue tetrazolium

OCT – optimum cutting temperature

PBS – phosphate buffered saline

PMSF – phenylmethylsulfonyl fluoride

Introduction

Autoimmune bullous diseases are chronic diseases of unknown origin characterized by blistering of the skin and associated with tissue-bound and circulating autoantibodies to structural components of the skin. Based on clinical and histopathological features, autoimmune bullous diseases may be divided into pemphigus-type and subepidermal autoimmune diseases. The first group of diseases includes blistering diseases characterized by intraepidermal blister formation due to the loss of adhesion of keratinocytes and is associated with autoantibodies to the intercellular junctions of keratinocytes. The second group of diseases is characterized by subepidermal blisters caused by the loss of attachment of basal keratinocytes to the underlying basement membrane and is associated with autoantibodies to the dermal-epidermal junction.

Autoantibodies in patients with pemphigus were shown to specifically react with desmogleins (Stanley *et al,* 1982; Amagai *et al,* 1991; Karpati *et al,* 1993; Wang *et al,* 1997) and desmocollins (Hashimoto *et al,* 1997) (desmosomal transmembrane cell-adhesion molecules of cadherin type), with plakoglobin (an intracellular protein of the plakin family) (Korman *et al,* 1989), and with acethylcholine receptors (Nguyen *et al,* 2000a; Nguyen *et al,* 2000b).

Over the past two decades, efforts have been made to identify antigens at the dermal-epidermal junction (DEJ) recognized by autoantibodies in patients with various forms of autoimmune subepidermal blistering diseases (**Figure 1**). Depending on the primary site of involvement, autoantibodies may bind to distinct DEJ molecules: the bullous pemphigoid (BP) antigens of 230kD (BP230) (Stanley *et al,* 1981) or 180kD (BP180) (Diaz *et al,* 1990; Giudice *et al,* 1992), plectin (Laffitte *et al,* 2001) and the α_3 (Domloge-Hultsch *et al,* 1992; Kirtschig *et al,* 1995), β_3 (Ghohestani *et al,* 2000b), and γ_2 (Nousari *et al,* 1999) subunits of laminin-5; the α_6 (Bhol *et al,* 2001) and β_4 (Tyagi *et al,* 1996; Bhol *et al,* 2000; Kumari *et al,* 2001) chains of $\alpha_6\beta_4$ integrin; a 168–170kD mucosal antigen (Ghohestani *et al,* 1996); a 200 kDa dermal autoantigen (Zillikens *et al,* 1996), type VII collagen (Woodley *et al,* 1984), and the α_5 chain of type IV collagen (Ghohestani *et al,* 2000a). DEJ

molecules that were identified as autoantigens in subepidermal blistering diseases are represented in **Figure 1**.

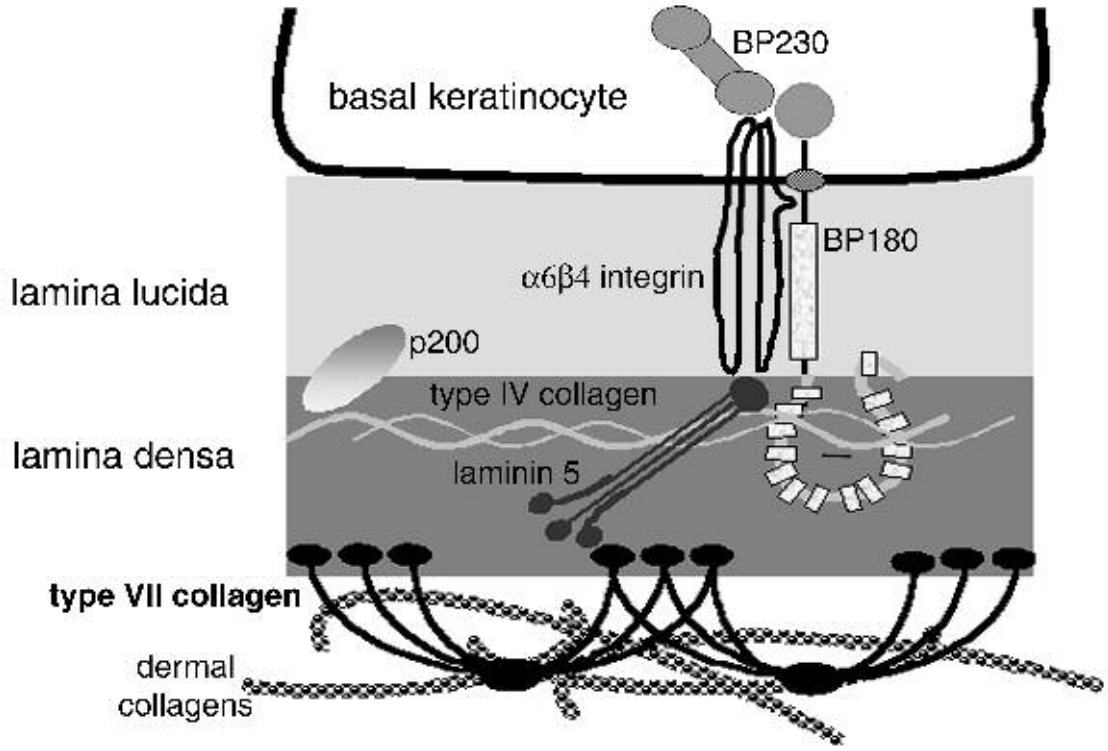

***Figure 1*. Schematic representation of autoantigens of the dermal-epidermal junction**. Structural components of the skin basement membrane that may function as autoantigens in subepidermal blistering diseases and their relative position are shown. Plectin and BP230 are intracellular hemidesmosomal proteins. BP230 interacts with BP180 and $\alpha_6\beta_4$ integrin which are transmembrane hemidesmosomal components. The α_6 chain of $\alpha_6\beta_4$ integrin interacts extracellularly with BP180. $\alpha_6\ \beta_4$ integrin is a known ligand for laminin 5 in the lower lamina lucida and lamina densa. P200 is a not yet fully characterized autoantigen localized to the lower lamina lucida. Beside other ubiquitous proteins like perlecan and nidogen (not shown), laminin 5 and type IV collagen form a network in the lamina densa. Laminin 5 binds to type VII collagen which represents the major component of anchoring fibrils.

Epidermolysis bullosa acquisita (EBA) is a rare chronic blistering disease of skin and mucous membranes characterized by subepidermal blisters and tissue-bound and circulating autoantibodies to the dermal-epidermal junction (DEJ). Type VII collagen, the main constituent of anchoring fibrils, was identified as the autoantigen of EBA (Woodley *et al,* 1984; Woodley *et al,* 1988).

EBA is a clinically heterogeneous disease and patients may present with an inflammatory or non-inflammatory phenotype. The first cases of a blistering disease with adult onset and features highly reminiscent of hereditary dystrophic epidermolysis bullosa were reported by Elliott more than 100 years ago (Elliott 1895). The mechanobullous, non-inflammatory form of EBA, was defined in 1971 and is characterized by extreme skin fragility, trauma-induced blisters and erosions localized to the extensor skin surface, healing with scars and milia (Roenigk *et al,* 1971). In addition to the mechano-bullous variant, several inflammatory subtypes of EBA were described, clinically mimicking bullous pemphigoid, linear IgA disease, or mucos membrane pemphigoid (Dahl 1979; Gammon *et al,* 1982a; Gammon *et al,* 1984b). Certain EBA patients present with inflammatory phenotype at the onset of the disease with overlapping or later evolving mechanobullous features (Gammon *et al,* 1984b; Stewart *et al,* 1991). EBA typically affects adults but juvenile cases were also reported (McCuaig *et al,* 1989; Bernard *et al,* 1995; Lacour *et al,* 1995; Callot-Mellot *et al,* 1997; Caux *et al,* 1997; Park *et al,* 1997; Schmidt *et al,* 2002). EBA is a rare disease occuring in approximately 5% of unselected patients with basement membrane zone antibodies (Zhu *et al,* 1990).

The disease is immunopathologically characterized by deposition of immunoreactants at the DEJ by direct immunofluorescence (IF) microscopy. The IgG and/or C3 deposits were shown to localize to the sublamina densa region of DEJ by direct immunoelectron microscopy (Yaoita *et al,* 1981). Indirect IF microscopy on 1M NaCl split-skin evidenced autoantibodies to the dermal versant of the DEJ in serum of EBA patients (Gammon *et al,* 1984a) which label the sublamina densa zone by indirect immunoelectronmicroscopy (Woodley *et al,* 1984). Altough the site of immunoreactants deposition is always lamina/sublamina densa region, in some EBA patients the cleavage plane localizes to the lamina lucida of the DEJ (Fine *et al,* 1989). EBA serum autoantibodies recognize the 290 kDa type VII collagen by immunoblotting with dermal extracts (Woodley *et al,* 1984) and immunoprecipitation with keratinocyte and fibroblast extracts (Stanley *et al,* 1985).

Type VII collagen, initially isolated from human chorioamniotic membranes, is an anti-parallel dimer recognized as the major constituent of anchoring fibrils (Bentz *et al,* 1983; Morris *et al,* 1986; Sakai *et al,* 1986; Keene *et al,* 1987). The gene

COL7A1, encoding for the human type VII collagen was mapped to the short arm of the chromosome 3 (Parente *et al,* 1991; Greenspan *et al,* 1993). The COL7A1 gene consists of 118 exons, more than any previously described gene (Christiano *et al,* 1994a). Type VII collagen is composed of three identical α chains, each consisting of a 145-kDa central collagenous triple-helical portion, flanked by a large 145-kDa amino-terminal non-collagenous domain (NC1), and a smaller 34-Kda carboxy-terminal non-collagenous domain (NC2) (Parente *et al,* 1991) (**Figure 2**). In the extracellular space, type VII collagen molecules form antiparallel tail-to-tail dimers stabilized by disulfide bonding through a small carboxy-terminal overlap (NC2), while a fragment of the NC2 domain is proteolitically removed (Bruckner-Tuderman *et al,* 1995; Chen *et al,* 2001).

The epitopes recognized by EBA sera were mapped to the NC1 domain of type VII collagen and autoantibodies in a minority of sera were also shown to react with triple-helical or NC2 domains (Woodley *et al,* 1988; Gammon *et al,* 1993; Lapiere *et al,* 1993; Tanaka *et al,* 1994; Chen *et al,* 2001; Schmidt *et al,* 2002), while exclusive reactivity to the triple-helical domain was documented in only three children with EBA (Tanaka *et al,* 1997).

The biological significance of type VII collagen is further substantiated by the existence of a hereditary form of dystrophic epidermolysis bullosa. In the skin of these patients type VII collagen shows altered expression. Evidence accumulated that genetic defects in the correct synthesis, secretion, or in the molecular assembly of type VII collagen cause the different clinical forms of dystrophic epidermolysis bullosa (Leigh *et al,* 1988; Bruckner-Tuderman *et al,* 1989; Bruckner-Tuderman *et al,* 1990; Smith and Sybert 1990; Christiano *et al,* 1993; Christiano *et al,* 1994b; Hovnanian *et al,* 1997). Interestingly, type VII collagen was found to have an elevated and topographically aberrant expression in the dermis of patients with systemic sclerosis under the regulatory action of transforming growth factor-beta (TGF-β). The presence of type VII collagen in the dermis may contribute to the tightly bound and indurated appearance of the affected skin in these patients (Rudnicka *et al,* 1994).

EBA is thought to be an antibody-mediated autoimmune disease. It has been suggested that EBA autoantibodies may disrupt the interaction of type VII collagen

with fibronectin or may interfere with the antiparallel dimer formation (Woodley *et al,* 1987; Chen *et al,* 2001). In addition, a pathomechanism of immune complex-mediated inflammation involving complement and leukocyte activation was proposed (Gammon *et al,* 1984c; Gammon *et al,* 1989; Mooney *et al,* 1992). However, there is a functional heterogeneity of *in situ* bound and circulating autoantibodies in EBA with respect to their complement-fixing ability and capacity to induce neutrophil chemotaxis and activation (Gammon and Briggaman 1987). Subclass distribution of polyclonal EBA autoantibodies was also shown not to correlate with their complement-fixing ability (Mooney and Gammon 1990; Bernard *et al,* 1991; Cho *et al,* 1998) nor to the clinical phenotype (Gandhi *et al,* 2000).

In other autoimmune blistering diseases, including pemphigus and anti-epiligrin/laminin 5 pemphigoid, the blister-inducing capacity of patients' autoantibodies has been demonstrated by passive transfer into neonatal BALB/c and severe combined immunodeficient (SCID) mice, respectively (Anhalt *et al,* 1982; Lazarova *et al,* 1996). Autoantibodies to β_4 chain of $\alpha_6\beta_4$ integrin from patients with ocular cicatricial pemphigoid were shown to induce subepidermal blisters in an *in vitro* conjunctival organ culture model (Chan *et al,* 1999). In addition, autoantibodies to α6 chain of $\alpha_6\beta_4$ integrin from patients with patients with oral pemphigoid produced subepidermal blisters when incubated with cultured normal human buccal mucosa (Bhol *et al,* 2001). While antibodies from patients with bullous pemphigoid do not cross-react with murine skin and do not induce blisters by passive transfer into neonatal mice, IgG from rabbits, immunized with recombinant murine type XVII collagen/BP180, led to a blistering disease in the mice that mimicked bullous pemphigoid (Liu *et al,* 1993). Recently, we demonstrated that autoantibodies to human type XVII collagen from patients with bullous pemphigoid induce dermal-epidermal separation in cryosections of human skin when co-incubated with leukocytes from healthy volunteers (Sitaru *et al,* 2002). However, the blister-inducing capacity of autoantibodies to type VII collagen has not yet been unequivocally demonstrated. Previous attempts to induce EBA by passive transfer of patients' autoantibodies into neonatal BALB/c mice (Shigemoto *et al,* 1988; Borradori *et al,* 1995) or human skin grafted onto SCID mice were not successful (Chen *et al,* 1992).

Aim of the study

The aim of the present study was to demonstrate the blister-inducing ability of autoantibodies to type VII collagen from serum of patients with EBA. Previously, sera from some EBA patients were shown to induce leukocyte recruitment to the DEJ using a leukocyte attachment assay (Gammon *et al,* 1984c). Modifying this assay, we incubated cryosections of human skin with IgG preparations from EBA patients and subsequently with leukocytes from healthy donors. We addressed the question, if autoantibodies from EBA sera, affinity-purified against recombinant type VII collagen, have the ability to trigger an immune complex-mediated inflammation leading to blister formation in the cryosections. In further experiments, we aimed at defining the epitopes targeted by the pathogenic autoantibodies, the ultrastructural site of blister formation, the leukocyte subpopulations that mediate this effect, and the role of the Fc portion of autoantibodies for dermal-epidermal separation in this model.

Materials and Methods

Antibodies

Serum samples were obtained from 16 patients with EBA prior to the initiation of treatment (**Table 1**). Nine out of the 16 patients presented with the non-inflammatory type of the disease and 5 with inflammatory EBA, while in 2 patients, the clinical data available did not allow a precise classification. Two patients (EBA13 and EBA14) suffered from the childhood variant of the disease (5 and 6 years, respectively), the remaining of the patients were adults (mean age 58 years). All EBA patients were characterized by 1) blisters on the skin; 2) linear deposits of IgG at the DEJ by direct IF microscopy; 3) circulating IgG autoantibodies binding to the dermal side of 1M NaCl-split human skin by indirect IF microscopy (titers ranging from 40 to 1280); and 4) immunoblot reactivity with type VII collagen extracted from dermis. By complement-fixation test on neonatal foreskin (Katz *et al,* 1976), 12 EBA sera fixed complement to the dermal-epidermal junction, whereas 4 sera did not. Serum samples from patients with bullous pemphigoid (n=20), pemphigus vulgaris (n=6), and from healthy donors (n=20) were used as controls. Murine mAbs to type IV collagen (clone CIV 22, IgG1) and to type VII collagen (clone LH7.2, IgG1) were purchased from Sigma (St. Louis, MO).

Table 1. Characteristics of EBA patients included in this study

Patient	IIF Titer[A]	CBT (C3)[B]	Type VII Collagen[C]	rNC1[D]
EBA1	40	-	+	+
EBA2	160	+	+	+
EBA3	320	+	+	+
EBA4	320	+	+	+
EBA5	160	+	+	+
EBA6	160	+	+	+
EBA7	320	+	+	+
EBA8	160	-	+	+
EBA9	160	+	+	+
EBA10	640	+	+	+
EBA11	160	+	+	+
EBA12	160	+	+	+
EBA13	1280	+	+	+
EBA14	640	-	+	+
EBA15	80	-	+	+
EBA16	320	-	+	+

[A]Antibody titer by indirect IF microscopy on 1M NaCl-split human skin. [B]Complement-fixing capacity as revealed by complement-fixing test on neonatal foreskin. [C]Immunoblot reactivity with full-lenght type VII collagen extracted from dermis. [D]Immunoblot reactivity with the recombinant NC1 domain of type VII collagen.

Construction of cDNA for the Type VII Collagen NC1 Domain

Amplification and cloning procedures were performed as described previously (Kromminga *et al,* 2000; Kromminga *et al,* 2002). Briefly, primers for polymerase chain reactions (PCR) were synthesized by Perkin-Elmer/Applied Biosystems (Foster City, CA). DNA sequence data of type VII collagen were retrieved from EMBL DNA

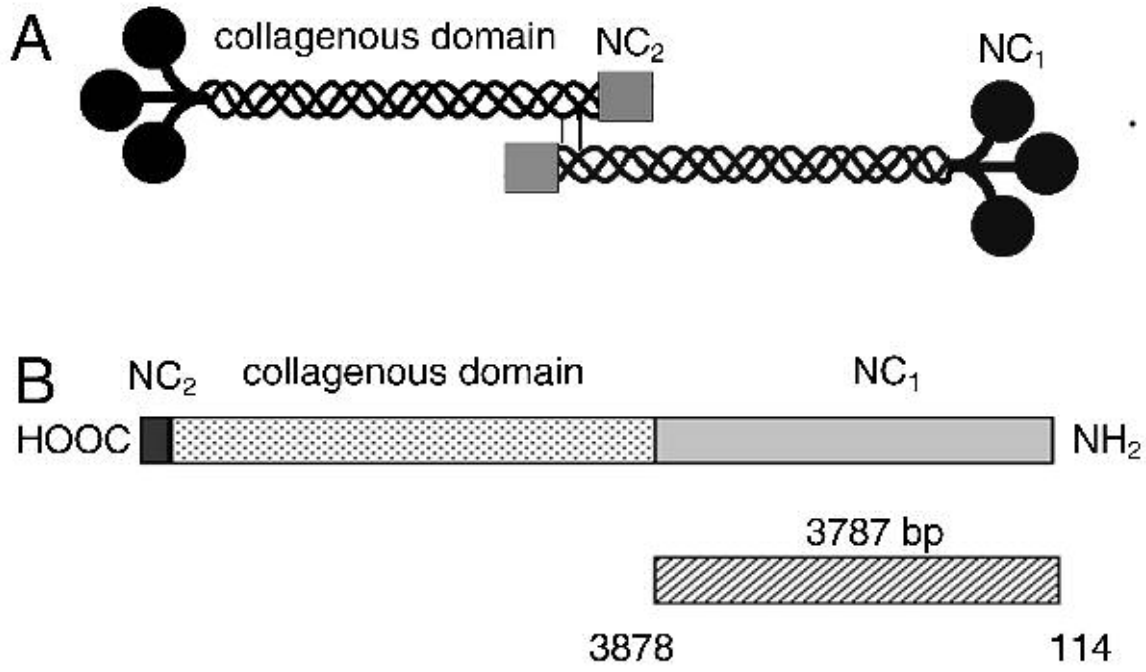

Figure 2. **Schematic diagram of type VII collagen.** (**A**) Type VII collagen molecule is a homotrimer composed of α chains. After secretion into the extracellular space, type VII collagen molecules form tail-to-tail dimers stabilized by disulfide bonding through a carboxy-terminal overlap. (**B**) Each α chain is composed of a central triple helical domain flanked by a large non-collagenous portion (NC1) at the N-terminus and by a smaller non-collagenous domain (NC2) at the C-terminus. The number of base pairs (bp) of the NC1-cDNA fragment corresponding to the NC1 domain recombinantly expressed is shown above the bar. Recombinant NC1 protein contains a hexahistidine moiety at the N-terminus.

data bank (Cambridge, UK) using the most recent accession number NM_000094.(Parente *et al,* 1991) The full-length 3787 bp NC1-cDNA (nt 114-3878) was generated by applying nested PCR on a cDNA pool generated by reverse transcription of total RNA from human keratinocytes. Restriction sites for Sal I and Hind III were introduced during the nested PCR using the primer pair p323 gataagctt atgacgctgcggcttctggtggccgcgctctg and p326 gatgtcgac ctggccctttggacaatacactgggcagggc. The NC1-cDNA was cloned into linearized pBBH2c (Invitrogen, Carlsbad, CA) resulting in the recombinant transfer vector pBBHNC1-FL. The presence of correct inserts was verified by DNA sequence analysis following the dideoxy method using a DyeDideoxy Terminator kit (Perkin Elmer) and a 377 DNA sequence analyzer (Perkin Elmer).

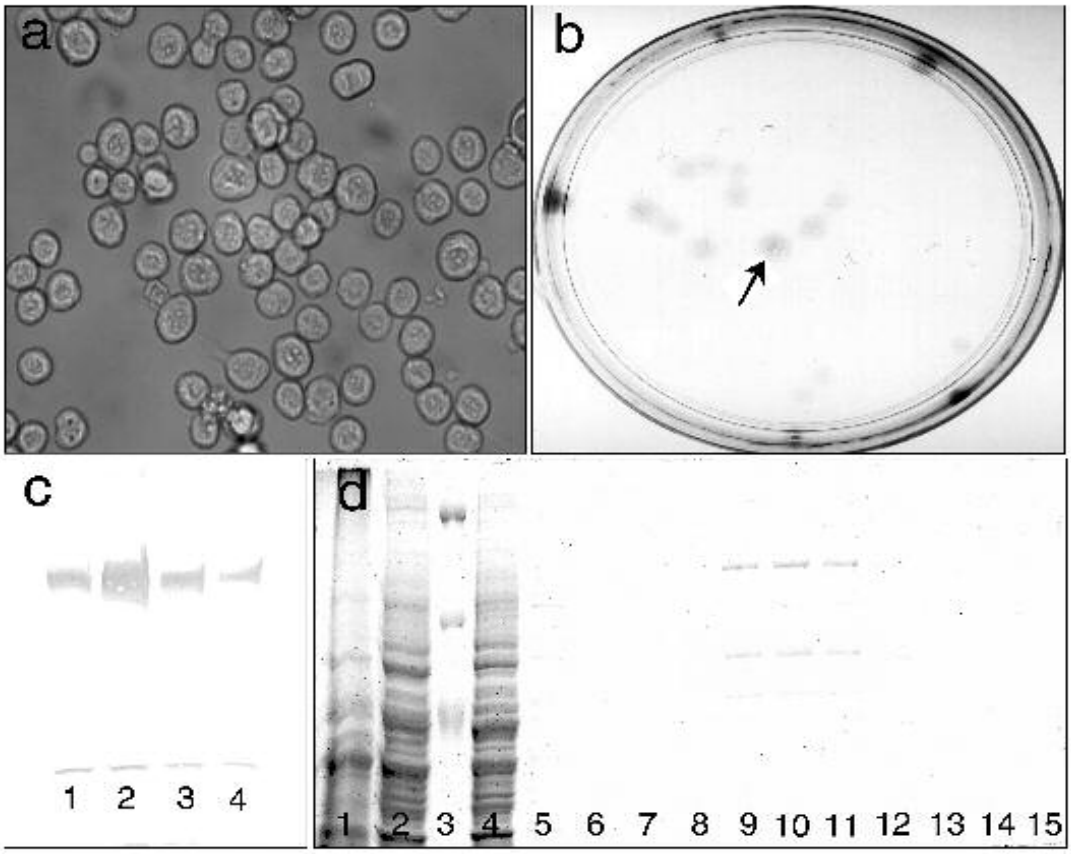

Figure 3. **Expression of the recombinant NC1 domain of type VII collagen in baculovirus-infected insect cells.** (**a**) Spodoptera frugiperda (Sf) 21 were co-transfected with the recombinant vector pBBHNC1-FL and linearized baculovirus AcMNPV DNA using the liposome technique. (**b**) The resulting recombinant baculovirus BV-NC1 was purified by plaque assay. A plaque indicates the presence of recombinant virus (arrow). (**c**) The ratio of virus copies to insect cells (i.e., multiplicity of infections, MOI) yielding the largest amount of recombinant protein was determined by infecting 2 x 10^5 insect cells with different dilutions of high-titer virus stock resulting in MOI of 1, 2, 3, and 4, respectively. The insect cell lysates were then analyzed by immunoblotting with monoclonal anti-RGSHHH-antibody (e.g., for this experiment a MOI of 2 resulted in best expression of recombinant NC1). (**d**) Under these optimized conditions, the expression of recombinant NC1 was scaled-up and the recombinant protein was purified from insect cell lysates by immobilized metalochelate affinity chromatography using Ni-NTA. The eluted fractions (*lanes 7-15*) were analyzed by SDS-PAGE and protein rich fractions were pooled (*lanes 9-11*). Molecular weight marker is shown in *lane 3*.

Heterologous Expression of Type VII Collagen NC1 in Sf21 Insect Cells

Spodoptera frugiperda 21 (Sf21) insect cells (Invitrogen) grown in Grace's insect medium (Invitrogen) were co-transfected with the recombinant vector pBBHNC1-FL

and linearized baculovirus AcMNPV DNA (Bac-N-Blue™, Invitrogen) using the liposome technique resulting in recombinant baculovirus BV-NC1 (**Figure 3**). For control experiments, insect cells were co-transfected with linear baculovirus DNA and transfer vector pBBH2c (lacking NC1-cDNA) resulting in recombinant baculovirus BV-0. Recombinant baculovirus clones were identified and purified by plaque assay. Recombinant NC1 and a vector-specific peptide of 52 amino acids including only the hexa-histidine tag and a sequence encoded by the multi-cloning site, but no NC1 sequence, were expressed by infecting the cells with recombinant baculovirus BV-NC1 or BV-0 at a multiplicity of infection of 5.0 and 4.0, respectively. Two days post-infection, the recombinant hexa-histidine tagged proteins were purified from Sf21 insect cells by immobilized metal chelate affinity chromatography using Ni-NTA agarose (Qiagen). For analysis of expression and purification of the recombinant protein, monoclonal anti-RGSHHH-antibody (Qiagen) was used (Kromminga *et al,* 2002). Protein concentrations were measured by Bradford assay (Biorad, Hercules, CA).

Immunoblot Analysis

Extracts of human dermis were prepared as described (Zillikens *et al,* 1996). Briefly strips of skin discarded from plastic surgery or alternatively foreskins were incubated in phosphate buffered saline (PBS) containing 1 M NaCl, 2 mM EDTA and 2mM PMSF for 48 hours at 4°C to separate epidermis and dermis. The epidermis was then removed using fine foreceps. Dermal proteins were extracted for 1 hour at room temperature using a buffer containing 12.5 mM Tris-HCL, 8 M urea, 0,1 M DL-Dithiothreitol, 2 mM EDTA 2% w/v SDS and a cocktail of protease inhibitors (all Sigma, Deisenhofen, Germany). The extract was centrifuged at 15.000 g for 10 minutes at 4°C, aliquoted and stored at –80°C until used. Recombinant NC1 or dermal extracts were fractionated by 8% and 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively, transferred to nitrocellulose, and analyzed by immunoblotting (Zillikens *et al,* 1999). Blots were blocked for 45 minutes in 5% solution of skimmed milk powder in Tris-buffered saline plus Tween-20 (TBST) buffer (0.02 M Tris-[hydromethyl)-aminomethane, 0.14 M NaCl, TWEEN-20 0.01%, pH 7.5, all Sigma). Sera from patients and healthy controls were diluted 1:100 and bound autoantibodies were visualized enzymatically using

peroxidase-conjugated rabbit anti-human IgG (1:500) (DAKO, Glostrup, Denmark). Type VII collagen in dermal extracts and recombinant NC1 were detected by the mouse monoclonal antibody LH7.2 (diluted 1:100 in 1% BSA TBST; Sigma). As second antibody HRP-conjugated rabbit anti-mouse antibody (1:500, DAKO) was used.

Affinity-Purification of Total IgG and IgG Specific for Type VII Collagen

IgG from serum of patients and controls was isolated using Protein G Sepharose Fast Flow affinity column chromatography following the instructions of the manufacturer (Pharmacia AB, Uppsala, Sweden). Antibodies from sera of EBA patients were affinity-purified using an AminoLink Plus immobilization kit following the manufacturer‘s instructions (Pierce, Rockford, IL). Recombinant protein encoded by BV-0 (lacking NC1) and recombinant NC1 protein (also including the vector-specific peptide) were covalently coupled to 4% beaded agarose at pH 10 and incubated with patient’s serum for 1 hour. Antibodies were eluted with 0.1 M glycine buffer (pH 2.5), neutralized with Tris-HCl, and concentrated under extensive washing with PBS (pH 7.2) using Ultrafree 15 filters (Millipore, Bradford, MA). Flow-through fractions were subjected to Protein G column chromatography and concentrations of IgG in flow-through and eluted fractions were determined by absorbance at 280 nm. Reactivity of both fractions was analyzed by indirect IF microscopy on salt-split skin and by immunoblotting with cell-derived type VII collagen or recombinant NC1.

Preparation of IgG F(ab')$_2$ Fragments

Immunoaffinity purified antibodies specific to type VII collagen were subjected to pepsin digestion (Parham 1983). Briefly, antibodies previously purified against recombinant NC1 were dialysed against 0.1M acetate buffer (pH 4) at 4°C for 4 hours. Pepsin in a concentration of 1:20 (pepsin to IgG) was added and the mixture was incubated at 37°C for 5 hrs with constant shaking. After the reaction was stopped with 50µl/ml 0.2M TRIS, the mixture was dialysed against PBS at 4°C overnight. Fc portions and undigested IgG were removed by protein G affinity chromatography and the F(ab‘)$_2$ preparation was concentrated by ultrafiltration. Completeness of fragmentation and reactivity of the F(ab‘)$_2$ preparations were tested

by indirect IF microscopy on 1 M NaCl split-skin using FITC labelled secondary antibodies specific to Fab (Sigma, St. Louis, MO) and Fc (Serotec Ltd, Oxford, UK) portions of IgG, respectively.

Peripheral Blood Leukocytes and Complement

Peripheral blood leukocytes from healthy volunteers were isolated by a sedimentation gradient medium containing sodium diatrizoate and dextran 500 (Nycomed, Oslo, Norway). Cells were harvested, washed twice in RPMI 1640 (Life Technologies, Karlsruhe, Germany) and resuspended in the same medium. The cell suspension was kept on ice and cell viability was tested using trypan blue; preparations with a viability greater than 95% were used. To determine the activation status of leukocytes, the cells were resuspended in medium containing 0.05% nitroblue tetrazolium (NBT; Roth, Karlsruhe, Germany) prior to incubation with the cryosections. Subsequently, unfixed sections were examined by light microscopy for the presence of dark blue formazan precipitates. Serum samples from healthy volunteers served as a source of human complement and were diluted 2-fold with RPMI (Gammon *et al,* 1980).

Treatment of Cryostat Sections

Neonatal human foreskin, obtained from routine circumcision, was washed in cold PBS, cut in pieces of 5 x 15 mm, embedded in optimum cutting temperature compound (Sakura Finetek Europe B.V., Zoeterwonde, Netherland), and stored at -80°C. Four cryosections of 6 µm were placed in the center of a Superfrost Plus microscope slide (Menzel-Gläser, Braunschweig, Germany). Sera from EBA patients and controls were heat-inactivated for 30 minutes at 56°C and diluted 2-fold in PBS. Prior to the treatment of skin sections, protein G affinity-purified IgG from EBA sera was diluted in PBS to an indirect IF titer of 80 and the monoclonal antibody LH7.2 was used at an indirect IF titer of 1000. When EBA sera were purified against recombinant type VII collagen NC1, flow-through and eluted fractions were adjusted to an IgG concentration of 0.5 mg/ml. Skin sections were washed with PBS for 5 minutes to remove embedding medium before incubation with 50 µl of diluted sera or antibody preparations (90 minutes at room temperature). After washing the sections

with PBS twice, chambers were prepared as described (Gammon *et al,* 1982b) and the leukocyte suspension, mixed 1:1 with either diluted fresh or heated serum or medium alone, was injected into the chambers. Incubation occured in a humidified air incubator containing 5% CO_2 for 3 hours at 37°C. Subsequently, chambers were disassembled, sections were washed in PBS for 10 minutes, air dried for 10 minutes, fixed in formaline, and stained with hematoxylin and eosin.

Results

Preparation of the Recombinant NC1 Domain of Type VII Collagen

The recombinant NC1 domain was expressed in insect cells infected with baculovirus and purified by immobilized metalochelate affinity chromatography using Ni-NTA agarose. Sera from 16 EBA patients and mAb LH7.2 reacted with the 145 kDa recombinant NC1 domain by immunoblotting, in contrast to sera from patients with bullous pemphigoid (n=20), pemphigus vulgaris (n=6), and from healthy controls (n=20). Typical patterns of immunoblot reactivity with recombinant NC1 are represented in **Figure 4**.

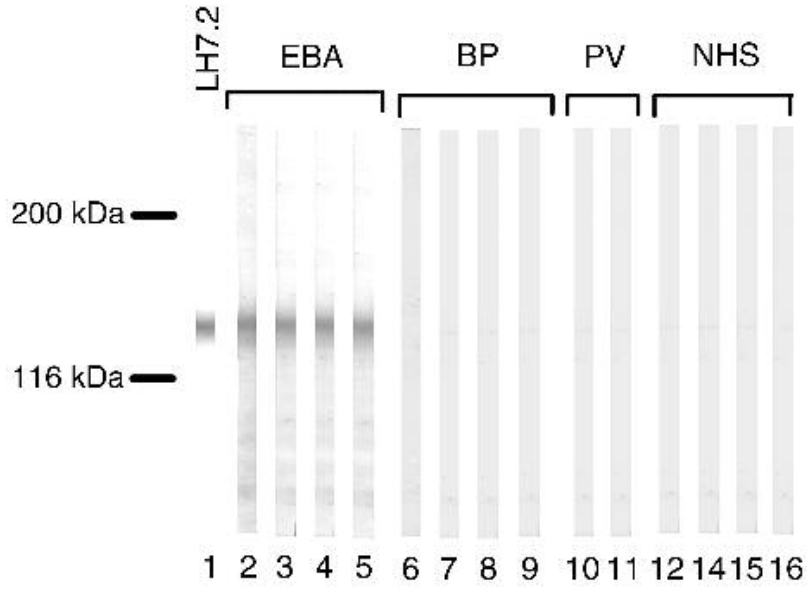

Figure 4. **Sera from patients with EBA, but not control sera, recognize the recombinant NC1 domain of type VII collagen by immunoblotting**. Like a monoclonal antibody to type VII collagen (lane 1), sera from EBA patients (*lanes 2-5*) strongly reacted with recombinant NC1 by immunoblot analysis. In contrast, sera from patients with bullous pemphigoid (*lanes 6-9*), pemphigus vulgaris (*lanes 10,11*), and healthy controls (*lanes 12-16*) show no reactivity with this recombinant protein.

Sera from EBA Patients Recruit Neutrophils to the Dermal-Epidermal Junction and Induce Subepidermal Splits in Cryosections of Human Skin

Cryosections of normal human skin were incubated with serum samples from EBA patients (n=16) and, subsequently, with leukocytes from healthy donors. IgG from EBA sera, but not from healthy controls, bound to the DEJ as revealed by indirect IF microscopy (**Figure 5a**). Addition of leukocytes resulted in the recruitment of leukocytes to the DEJ in cryosections that had been incubated with EBA sera, but not in those incubated with sera from controls (**Figure 5b**). We usually observed more background attachment of leukocytes when the sections were incubated with EBA sera compared with sections that were treated with control sera. To investigate the time-dependency of neutrophil attachment and blister formation, we incubated the cryosections, that had been treated with sera from patients EBA8 and EBA11, with leukocytes for 30, 60, 90, 120 and 180 minutes, respectively. Leukocyte attachment to the DEJ was strongest after an incubation time of 60 minutes (covering 65% of the length of the DEJ) and declined subsequently (30%, 14%, and almost no binding at 90, 120, and 180 minutes incubation, respectively). Dermal-epidermal separation was first seen after an incubation time of 60 minutes (on 10% of the length of the DEJ); with longer incubation times, the extent of dermal-epidermal separation increased and reached its maximum after 120 minutes (dermal-epidermal separation on 60% of the length of the DEJ) (**Figure 5e**). No dermal-epidermal separation was seen in cryosections treated with control sera (**Figure 5f**). The extent of dermal-epidermal separation was dependent on the number of leukocytes. At a 2 hour incubation, splits were seen when we used 10^7 cells/ml. The separation became larger with the use of greater numbers of neutrophils. With numbers exceeding $3x10^7$/ml, the extent of splits did not further increase, but both epidermis and dermis were non-specifically and increasingly damaged. When we used leukocyte numbers below 10^7/ml or when we omitted leukocytes, this abolished blister induction completely. Under the optimized assay conditions (incubation with $3x10^7$ leukocytes for 120 minutes), 14 out of 16 EBA sera induced dermal-epidermal separation, while serum from EBA13 and EBA14 did not. End point titers of immunoblot reactivity with recombinant NC1 of those EBA sera that induced a dermal-epidermal separation ranged from 500 to 5000. End point titers of immunoblot reactivity of sera EBA13 and EBA14, that were not able to

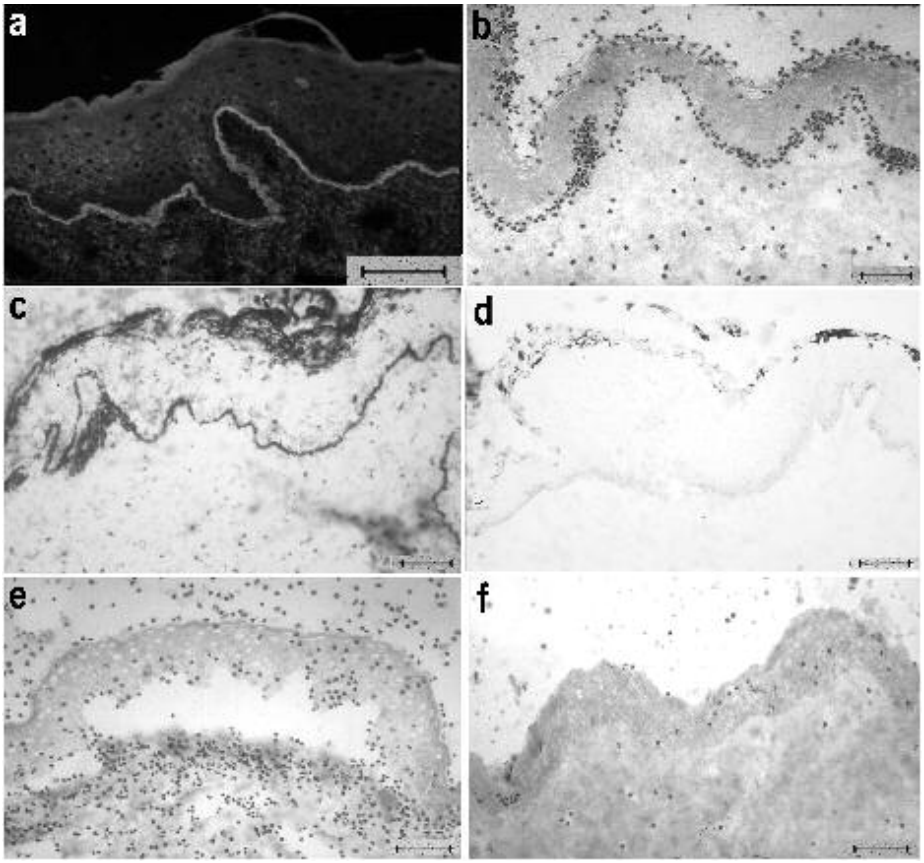

***Figure 5*. Serum from an EBA patient recruits neutrophils to the dermal-epidermal junction and induces subepidermal splits.** (**a**) Cryosections of normal human skin, incubated with EBA serum, show IgG deposits at the dermal-epidermal junction by indirect IF microscopy. (**b**) Subsequent addition of leukocytes leads to neutrophil recruitment to the dermal-epidermal junction. (**c**) Activation of neutrophils, as revealed by reduction of NBT to formazan (dark precipitates), is induced by EBA serum, (**d**) but not by normal human serum. (**e**) With longer incubation times, subepidermal splits develop in the cryosection that were treated with EBA serum. (**f**) In contrast, serum from a healthy donor does not induce neutrophil recruitment or dermal-epidermal separation. Bar, 40 µm.

induce separation, were 1000 and 2000, respectively. To address the question if the addition of complement augments the extent of dermal-epidermal separation in this model, we incubated cryosections, that were previously treated with EBA sera, with 1) leukocytes and fresh serum, 2) leukocytes and complement-inactivated (heated) serum, or 3) with leukocytes alone. Interestingly, the addition of complement did not increase the extent of dermal-epidermal separation in the cryosections compared with the extent observed when complement was heat-inactivated or omitted completely. In the presence of complement we observed neutrophil attachment to

the stratum corneum in all cryosections irrespective of whether they were incubated with serum from patients or controls. Since complement did not play a role in this model, further experiments did not include fresh serum as a source of complement.

Leukocytes at the Dermal-Epidermal Junction are Activated

To determine if leukocytes that attach to the DEJ become activated, we incubated cryosections, that had been treated with patients' sera (EBA3, EBA10, and EBA16), with leukocytes in the presence of NBT. After a 30 minute incubation, dark blue precipitates of reduced NBT (formazan) were found at both DEJ and stratum corneum (**Figure 5c**). In sections treated with normal human serum, no recruitment of cells to the DEJ was observed and blue precipitates were restricted to the stratum corneum (**Figure 5d**).

Granulocytes, but not Mononuclear Cells, are Required for Split Formation Induced by Autoantibodies from EBA Patients in Cryosections of Human Skin

To dissect the role of different leukocyte subpopulations in the split-formation induced by EBA autoantibodies, granulocytes and peripheral blood mononuclear cells from healthy donors were separated by gradient centrifugation. The preparations were used at a density of 3 x 10^7 cells/ml. When incubated with the

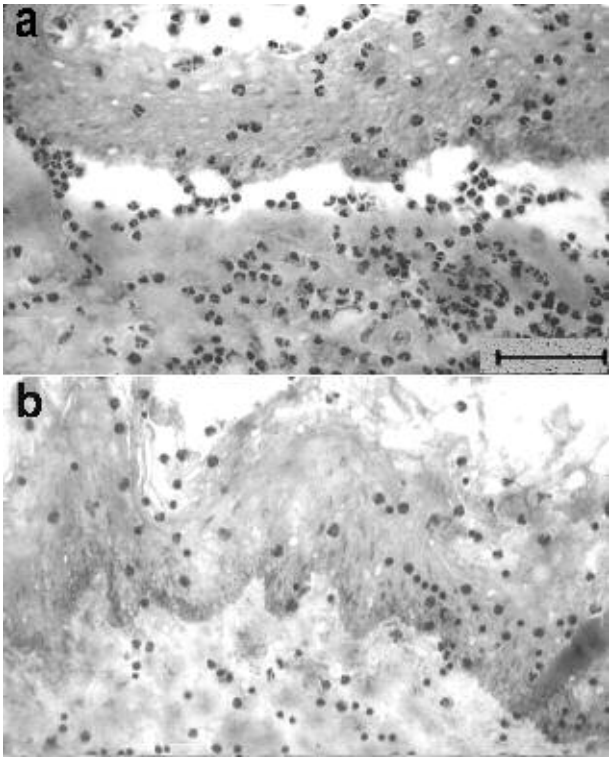

Figure 6. **Granulocytes, but not mononuclear cells, are required for split induction by autoantibodies from EBA patients in cryosections of human skin**. (**a**) Granulocytes, that were isolated from the blood of healthy donors, mediate subepidermal cleavage in cryosections treated with an EBA serum. (**b**) In contrast, mononuclear cells are not recruited to the DEJ and do not cause subepidermal cleavage.

cryosections that had been treated with serum from EBA3, EBA10, and EBA16, only granulocytes (**Figure 6a**), but not mononuclear cells (**Figure 6b**), were recruited to the dermal-epidermal junction and induced subepidermal splits.

The Cleavage Plane of Experimentally Induced Splits Localizes to the Lamina Lucida Zone of the Dermal-Epidermal Junction

To study the level of split formation, we stained cryosections, that had previously been incubated with sera from EBA patients and leukocytes, with monoclonal antibodies to human BP230 (1E5), type IV collagen, and laminin 5. Antibodies to BP230 stained the roof of the split, whereas those to both type IV collagen and laminin 5 labeled the dermal side demonstrating that the level of blister formation localizes to the lamina lucida of the DEJ (**Figure 7**).

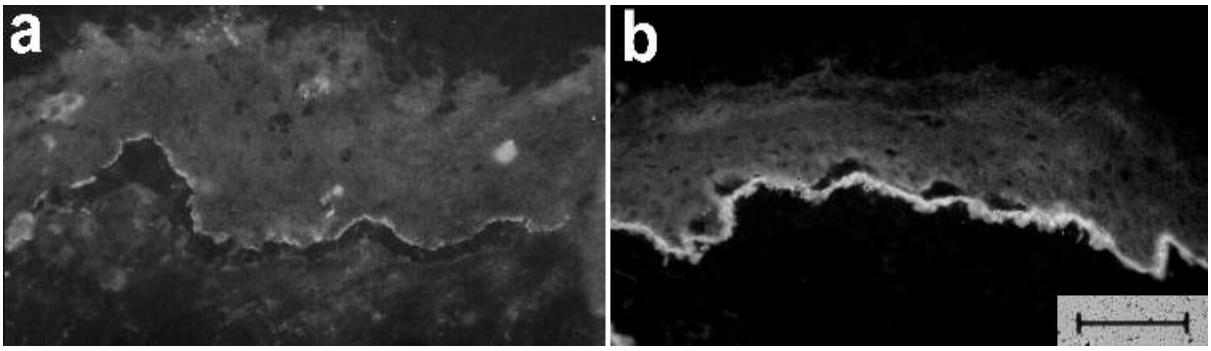

Figure 7. **By antigen mapping, experimentally induced splits localise to the lamina lucida of the dermal-epidermal junction.** Cryosections, that had been previously incubated with EBA sera and leukocytes, were stained with monoclonal antibodies to BP230 and laminin 5. Monoclonal antibody E5 to BP230 bound to the epidermal side of the cleft (**a**), whereas GB3 antibody to laminin 5 stained the dermal side (**b**) demonstrating that skin cleavage localizes to the lamina lucida zone of dermal-epidermal junction. *Scale bar*: 40 µm.

IgG Purified from Serum of EBA Patients Retains its Blister-Inducing Ability

To confirm the results obtained with patients' sera, we purified the IgG fractions of 5 EBA sera by Protein G column chromatography (**Table 2**). IgG preparations from patients and controls were used at the same IgG concentration. When incubated with the cryosections, IgG from EBA patients, in contrast to IgG from controls, induced subepidermal splits.

Table 2. Characterization of affinity-purified IgG preparations[A]

Patient	Concentration[B] (mg/ml)	IIF Titer[c]	Type VII collagen[D]	rNC1[E]
EBA4	22.5	160	+	+
EBA5	17.5	80	+	+
EBA7	25	320	+	+
EBA8	62.5	640	+	+
EBA11	21.3	160	+	+

[A]Total IgG fractions were isolated from human sera using protein G affinity column chromatography. [B]Concentrations of affinity-purified IgG was determined using absorbance at 280 nm. [c]Antibody titer by indirect IF microscopy on 1M NaCl-split human skin. [D]Immunoblot reactivity with full-lenght type VII collagen extracted from dermis. [E]Immunoblot reactivity with the recombinant NC1 domain of type VII collagen.

Antibodies Specific to the NC1 Domain of Type VII Collagen Induce Subepidermal Cleavage in Cryosections of Human Skin

In order to characterize the specificity of pathogenic autoantibodies from EBA sera, we purified antibodies from 5 EBA patients against a recombinant form of type VII collagen. Total IgG from the flow-through fractions was further purified by Protein G column chromatography. The reactivity and specificity of antibodies eluted from NC1 coupled to agarose and of IgG purified from the flow-through fractions are summarized in **Table 3** and representative patterns are shown in **Figure 8**. Importantly, when incubated with the cryosections, eluted autoantibodies specific to type VII collagen induced subepidermal splits (**Figure 8Ca**), whereas IgG depleted of reactivity to the NC1 domain of type VII collagen lost its blister-inducing ability (**Figure 8Cb**).

Table 3. Characterization of immunoaffinity-purified immunoglobulin preparations

Patient	IgG Preparation	Concentration[C] (mg/ml)	IIF Titer[D]	Type VII collagen[E]	rNC1[F]
Purification against recombinant NC1 encoded by BV-NC1[A]					
EBA4	Eluted fraction	2.7	320	+	2000[G]
	Flow-through fraction	21	-	-	-
EBA5	Eluted fraction	0.68	160	+	2000[G]
	Flow-through fraction	3.72	-	-	-
EBA6	Eluted fraction	2.56	160	+	2000[G]
	Flow-through fraction	2.2	-	-	-
EBA7	Eluted fraction	2.81	160	+	1000[G]
	Flow-through fraction	2.11	-	-	-
EBA8	Eluted fraction	0.68	320	+	5000[G]
	Flow-through fraction	39	-	-	-
Purification against the vector-specific peptide encoded by BV-0 [B]					
EBA 7	Eluted fraction	0.01	-	N.D.	-
	Flow-through fraction	10	320	N.D.	+
EBA8	Eluted fraction	0	-	N.D.	-
	Flow-through fraction	12	160	N.D.	+
EBA11	Eluted fraction	0.009	-	N.D.	-
	Flow-through fraction	8	160	N.D.	+

[A]Antibodies to type VII collagen from patients‘ sera were purified against recombinant NC1 covalently coupled to an agarose matrix. [B] Patients‘ sera were purified against Ni-NTA-purified lysate of Sf21 insect cells infected with BV-0. [C]Concentrations of affinity-purified antibodies were determined using absorbance at 280 nm. [D]Antibody titer by indirect IF microscopy on 1M NaCl-split human skin. [E]Immunoblot reactivity with full-lenght type VII collagen extracted from dermis. [F]Immunoblot reactivity with the recombinant NC1 domain of type VII collagen. [G]End point titers of immunoblot reactivity with recombinant NC1.

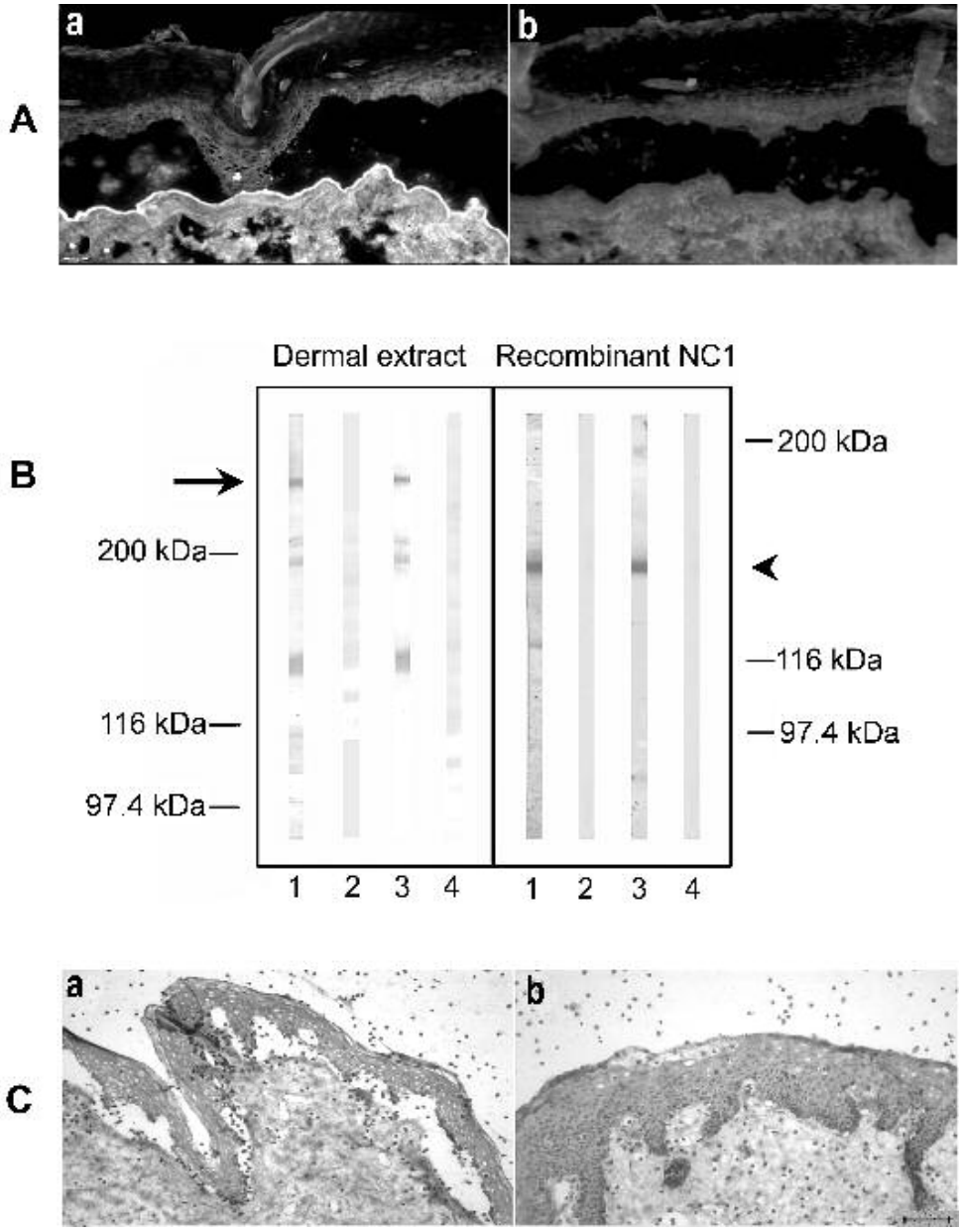

***Figure 8*. Immunoadsorption of EBA serum against the recombinant NC1 domain of type VII collagen abolishes split induction.** Autoantibodies to type VII collagen from EBA serum were purified using a recombinant form of the NC1 domain covalently coupled to an agarose matrix. (**A**) When analyzed by indirect IF microscopy (**a**) IgG eluted from recombinant NC1 retains reactivity with the dermal side of NaCl-split human skin, (**b**) in contrast to IgG from the flow-through fraction that completely lost IF reactivity. (**B**) By immunoblotting, IgG autoantibodies eluted from the column (*lane 3*) recognized, like a serum sample from the same patient (*lane 1*) both full-lenght type VII collagen extracted from dermis (arrow) and a recombinant form of its NC1 domain (arrowhead). In contrast, the flow-through fraction (*lane 2*) or normal serum (*lane 4*) showed no reactivity. (**C**) When incubated with the cryosections, (**a**) the patient's autoantibodies specific to type VII collagen NC-1, induce dermal-epidermal separation, (**b**) while IgG depleted of reactivity to the NC1 domain (flow-through fraction) does not . Bar, 40 µm.

To exclude the possibility that baculovirus- or insect cell-specific proteins other than recombinant NC1 non-specifically pre-adsorb the blister-inducing capacity of EBA sera, we purified 3 sera (EBA7, EBA8, and EBA11) against the recombinant vector-specific peptide, encoded by BV-0, not containing any NC1 sequence. Eluted fractions of this purification were non-reactive by indirect IF microscopy on salt-split skin and by immunoblotting with recombinant NC1 (**Figure 9Aa, B *lane 3***), whereas IgG from the flow-through (preadsorbed serum) stained the dermal side of salt-split skin by indirect IF microscopy (**Table 3, Figure 9Ab**) and reacted with recombinant NC1 by immunoblotting (**Figure 9B, *lane 2***). Both eluted and flow-through fractions were then incubated with the cryosections followed by incubation with leukocytes. Eluted fractions did not cause subepidermal splits, in contrast to flow-though fractions that were still pathogenic (**Figure 9C**). These results demonstrate that immunoadsorption of type VII collagen-specific antibodies was only achieved with recombinant NC1, but not with other proteins produced by baculovirus-infected insect cells.

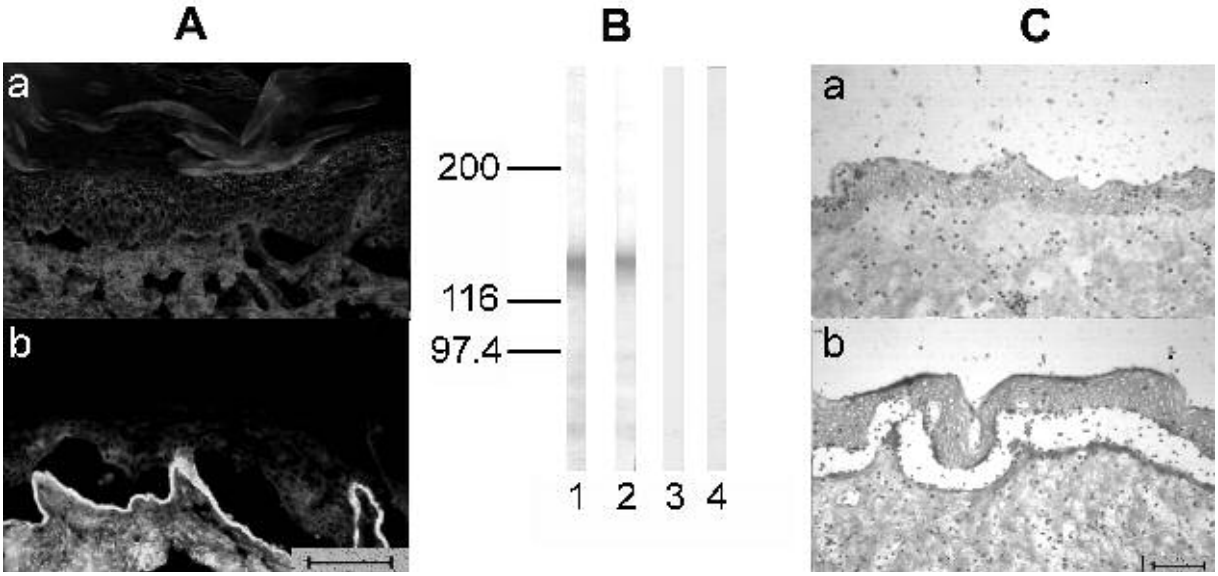

Figure 9. **Preadsorption of EBA serum against proteins from insect cells infected with recombinant BV-0, lacking NC1-cDNA, does not abolish its blister-inducing capacity.** EBA serum was purified against proteins from insect cells infected with recombinant BV-0 coupled to an agarose matrix. (**A**) By indirect IF microscopy, (**a**) IgG eluted from the column that completely lost indirect IF reactivity, (**b**) in contrast IgG from the flow-through fraction stained the dermal side of salt-split skin. (**B**) By immunoblotting, like the original serum (*lane 1*), the flow-through fraction (*lane 2*), reacted with recombinant NC1, while the eluted fraction (*lane 3*), like normal human serum (*lane 4*), was unreactive. (**C**) When incubated with cyosections, (**a**) in contrast to the eluted fraction, (**b**) the flow-through fraction retained its ability to induce blisters. Bar, 40 µm.

Autoantibodies to Type VII Collagen Induce a Dose-Dependent Dermal-Epidermal Separation in The Cryosections

To study the dependency of the extent of split formation on autoantibody reactivity to type VII collagen, we incubated the cryosections with different dilutions of both EBA sera and immunoaffinity-purified antibody preparations followed by incubation with leukocytes. As shown in **Table 4**, EBA sera and NC1-specific antibody preparations with higher indirect IF titers induced more extensive DEJ separation compared with preparations of lesser reactivity.

Table 4. Serum samples and NC1-specific autoantibodies from EBA patients induce a dose-dependent dermal-epidermal separation in cryosections of human skin

	EBA sera				**NC1-specific antibody preparations[A]**		
Patient	Dilution	IIF Titer[B]	DES (%)[C]	Patient	Dilution	IIF Titer[B]	DES (%)[C]
EBA8	1:2	80	70	EBA8	1:4	80	65
	1:4	40	60		1:16	20	40
	1:16	10	40		1:64	5	5
	1:160	1	10		1:320	1	0
EBA11	1:4	80	60	EBA11	1:2	80	75
	1:8	40	55		1:8	20	50
	1:32	10	30		1:32	5	30
	1:320	1	5		1:160	1	10

[A]Antibodies specific to NC1 were purified from the serum of 2 EBA patients by immunoaffinity column chromatography and incubated with cryosections of human skin followed by a 2 h incubation with human leukocytes. [B]Titer of antibodies by indirect IF microscopy on 1M NaCl-split human skin. [C]Length of DEJ with separation in relation to total length (percent).

A Monoclonal Antibody to the NC1 Domain of Type VII Collagen Induces Subepidermal Splits in the Cryosections

MAb LH7.2, directed to an epitope on the central portion of the NC1 domain of type VII collagen induced dermal-epidermal separation of the cryosections (**Figure 10a**). In contrast, a mAb to type IV collagen, that belonged to the same isotype and was used at the same IgG concentration (0.1 mg/ml), was not pathogenic (**Figure 10b**). The extent of split formation induced by mAb LH7.2 was less pronounced when compared with patients' autoantibodies to type VII collagen and split-induction required a longer incubation time with leukocytes (3 h).

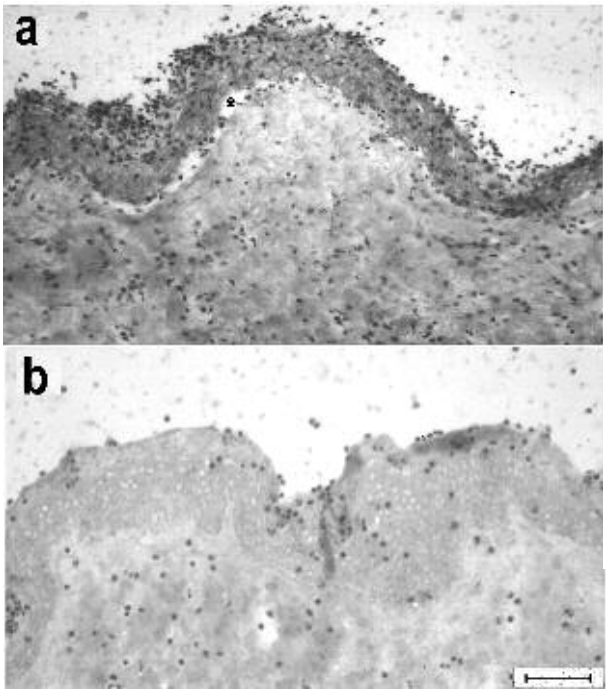

Figure 10. **A mAb to the NC1 domain of type VII collagen induces subepidermal splits in cryosections of human skin.** (**a**) Incubation of cryosections with mAb LH7.2, directed to the NC1 domain, and subsequently with leukocytes leads to dermal-epidermal separation, (**b**) in contrast to a mAb to type IV collagen that was used at the same IgG concentration. * Asterisk indicates the cleavage. Bar, 40 µm.

F(ab')2 Fragments of Blister-Inducing Autoantibodies Lose their Pathogenicity

To address the question, if the Fc portion of autoantibodies to type VII collagen is of importance for blister induction in our *in vitro* model, we prepared F(ab')$_2$ fragments of antibodies from EBA serum that had been eluted from recombinant NC1 coupled to agarose. F(ab')$_2$ fragments, lacking the Fc portion of the antibody, labeled the dermal side of NaCl-split skin by indirect IF microscopy (**Figure 11a, b**). Interestingly, F(ab')$_2$ preparations, although used at the same indirect IF titer (80) as unfragmented

IgG, failed to recruit neutrophils to the DEJ and to induce subepidermal cleavage when incubated with the cryosections (**Figure 11c, d**).

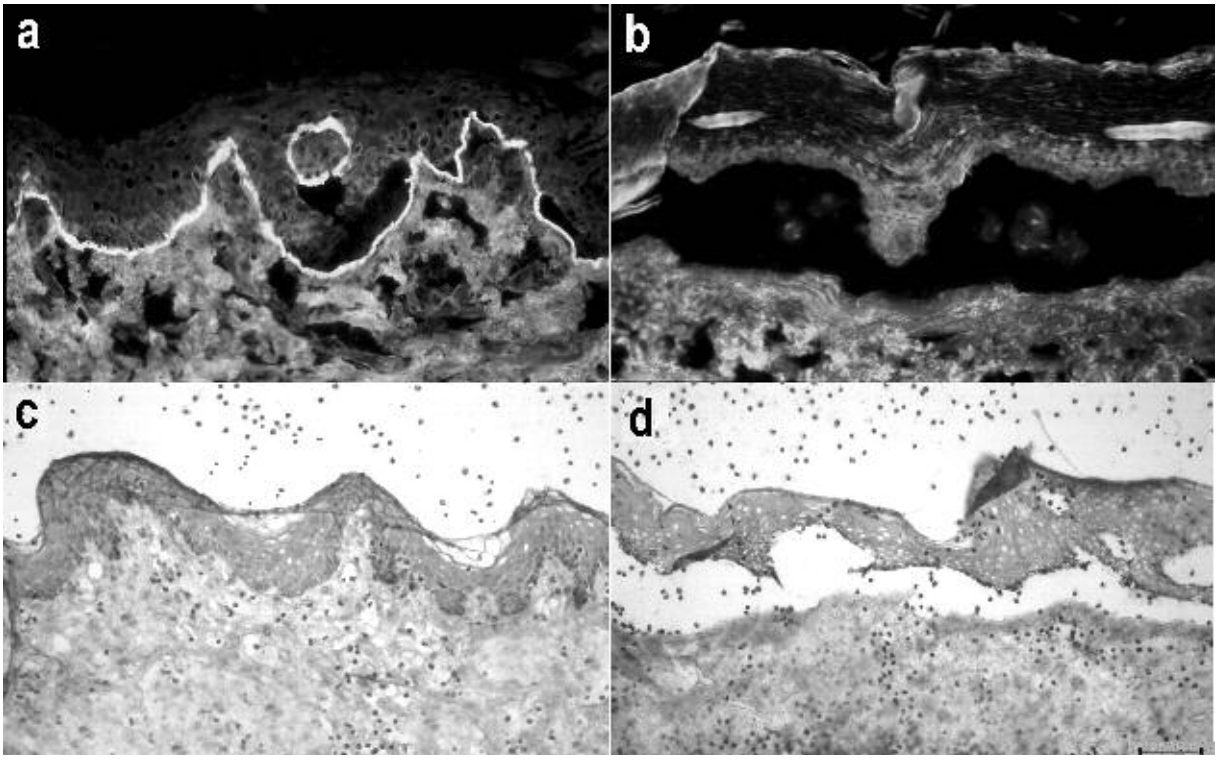

Figure 11. **Dermal-epidermal separation is dependent on the Fc portion of autoantibodies to type VII collagen.** Patient's autoantibodies, that had been purified against recombinant NC1 of type VII collagen, were subsequently subjected to pepsin digestion. (**a**) Resulting $F(ab')_2$ fragments labeled the dermal side of 1M NaCl-split skin by indirect IF microscopy using an anti-Fab FITC-conjugated secondary antibody, (**b**) whereas no staining was observed using a an Fc-specific conjugate. (**c**) $F(ab')_2$ fragments failed to induce blisters, (**d**) in contrast to unfragmented autoantibodies to type VII collagen. Bar, 40 µm.

Discussion

In this study, we show the capacity of autoantibodies to type VII collagen from EBA patients and of a monoclonal antibody to this protein to trigger an Fcγ-dependent inflammation leading to blister formation in cryosections of human skin. The pathogenicity of autoantibodies from patients with autoimmune blistering diseases directed to several structural components of the skin, including type XVII collagen, $\alpha_6\beta_4$ integrin, and laminin 5 had been previously demonstrated. In contrast, previous attempts to induce EBA by passive transfer of patients‘ autoantibodies into neonatal mice (Shigemoto *et al,* 1988; Borradori *et al,* 1995) or human skin grafted onto SCID mice had failed (Chen *et al,* 1992).

In a first set of experiments, we show that serum from EBA patients, in contrast to serum from healthy donors, induces recruitment and activation of neutrophils at the DEJ of the cryosections and, subsequently, dermal-epidermal separation. The development of subepidermal cleavage in cryosections, that strongly adhere to the glass slide, might be due to a “natural tension” within the DEJ. Indeed, bundles of elastic microfibrils of the lamina fibroreticularis anchor the basal lamina to dermal elastic fibres (Kobayashi 1977). Cleavage of components of the anchoring complexes by leukocyte-derived proteases may release this tension resulting in a recoil of elastic fibres and retraction of the dermis from the epidermis.

Complement was not required for split induction in this model. The group of EBA patients included in the present study was heterogeneous with respect to the complement-activation capacity of their serum. Serum from 5 of 16 patients did not fix complement to the dermal-epidermal junction as detected by indirect IF microscopy, in contrast to the remaining EBA sera. When complement was heat-inactivated or ommited completely, this did not affect the split induction by EBA sera in our *in vitro* assay. These observations confirm previous findings (Gammon and Briggaman 1987; Mooney and Gammon 1990; Gandhi *et al,* 2000) and suggest that complement activation is not likely to play a major pathogenic role in the blister formation at least in a subgroup of patients with EBA. However, in the cryosection model, in contrast to the *in vivo* situation, the leukocytes do not have to migrate

along a chemotactic gradient from blood vessels to the DEJ. Instead, by incubating the cryosections with leukocytes, the cells are placed in close contact with the DEJ. Therefore, the role of complement in the blister formation in EBA patients cannot be addressed using this model.

When fresh serum was added as a source of complement, we regularly found strong attachment of neutrophils to the stratum corneum of the cryosections, irrespective of whether the sections were incubated with serum from patients or controls. This observation, that we also made in previous studies using this *in vitro* assay (Sitaru *et al,* 2002), is in line with the finding that corneocytes may activate the alternative complement pathway thus mediating the adhesion of leukocytes to serum-treated stratum corneum in an antibody-independent manner (Terui *et al,* 1989; Terui *et al,* 1995). When fresh serum was ommited or replaced by heat-inactivated serum, we did not observe a major neutrophil adhesion to stratum corneum.

Of the 16 EBA sera we tested, 2 did not induce a split. Interestingly these sera came from 2 children and showed high reactivity by both indirect IF microscopy and immunoblotting of recombinant NC1. One may speculate that in these 2 patients, another pathogenic mechanism accounts for blister formation, which, in contrast to the majority of EBA sera, cannot be reproduced in our *in vitro* model.

In subsequent experiments, our findings with crude EBA serum were confirmed with the use of IgG purified from patients‘ sera. In contrast to IgG from healthy controls, patients’ IgG, when used at the same concentration, caused dermal-epidermal separation in the cryosections.

In the next set of our experiments we sought to determine the ultrastructural level of experimentally induced splits by antigen mapping. Interestingly, we found that the cleavage plane localizes to the lamina lucida of the DEJ. It has been previously shown that the level of blister formation does not always co-localize with the site of deposition of immunoreactants, for example in patients with dermatitis herpetiformis (Klein *et al,* 1983; Smith *et al,* 1992). The lamina lucida was viewed as a mechanical “locus minoris resistentiae” of the DEJ. The first electron microscopic studies of EBA patients, that belonged to the classical type of EBA, indicated the sublamina densa as the level of blister formation (Gibbs and Minus 1975); subsequent observations

showed that in the majority of the EBA patients the cleavage plane occurs within the lamina lucida also showing some correlation with the presence of neutrophil infiltration (Fine *et al,* 1989; Honoki *et al,* 1998). Double sublamina densa and lamina lucida localized split was also documented in one patient (Fine *et al,* 1989). These observations support the involvement of two different pathogenic mechanisms of blister formation in EBA that may act independently or occasionally in combination. By binding to type VII collagen, EBA autoantibodies, on one hand, may disrupt the interaction of type VII collagen with fibronectin or may interfere with the antiparallel dimer formation leading to blister formation within the sublamina densa. On the other hand, the formed IC may mediate recruitment and activation of neutrophils leading to a release of proteases and blister formation within the lamina lucida of the DEJ. Most likely, the level of cleavage within the sublamina densa may correlate with scarring and milia formation as typically seen in mechano-bullous form of EBA, while intra-lamina lucida blister formation may be found in blisters not associated with scarring and milia as seen in the inflammatory form of EBA. However, these 2 variants of EBA may represent only ends of a continuous spectrum and the individual clinical features may be determined by the different involvement of these two mechanisms.

Sufficient clinical data was available for 14 out of 16 patients included in the present study to diagnose these as non-inflammatory or inflammatory EBA. Nine belonged to the non-inflammatory and the remaining 5 patients to the inflammatory type. Serum from all nine non-inflammatory patients and from 3 patients with inflammatory disease caused dermal-epidermal separation in the cryosections. Further studies, based on a larger number of patients, will have to show if serum from non-inflammatory patients really have a greater split-inducing potential compared to serum from patients with inflammatory EBA.

The NC1 domain has been characterised as the immunodominant region of type VII collagen that is targeted by the majority of EBA sera (Gammon *et al,* 1993; Lapiere *et al,* 1993; Tanaka *et al,* 1994; Chen *et al,* 2001). To characterise the specificity of split-inducing autoantibodies in these sera, we produced a recombinant form of the NC1 domain expressed in baculovirus-infected insect cells. After purification by metalochelate affinity chromatography, the recombinant protein was used for immunoblotting with a monoclonal antibody to type VII collagen as well as serum

samples from patients with EBA, with other autoimmune bullous diseases and from healthy controls. Autoantibodies from serum of EBA patients were purified against recombinant NC1 that was covalently coupled to an agarose matrix. Importantly, antibodies eluted from the NC1 column retained their blister-inducing capacity, while IgG that was depleted of reactivity to NC1 lost its ability to recruit neutrophils and to induce blisters. To exclude the possibility that proteins produced by baculovirus-infected insect cells others than recombinant NC1 were responsible for pre-adsorbing the blister-inducing capacity of EBA sera, we purified serum from EBA patients against the recombinant vector-specific peptide, encoded by BV-0, not containing any NC1 sequence. These pre-adsorbed fractions retained the blister-inducing ability of original EBA sera demonstrating that the pre-adsorption procedure was antigen-specific.

In the next set of experiments we studied the dependency of the extent of dermal-epidermal separation on autoantibody reactivity to type VII collagen. We show that EBA sera and NC1-specific antibody preparations with higher indirect IF titers induced more extensive DEJ separation compared with preparations with lesser reactivity. This finding suggests that clinical activity of the disease (i.e., the extent of skin blistering) may correlate with the levels of autoantibody reactivity to type VII collagen in serum of patients with EBA.

The pathogenic relevance of the NC1 region was further supported by the finding that mAb LH7.2, directed to an epitope on the central portion of the NC1 domain of type VII collagen, in contrast to a mAb to type IV collagen, also induced subepidermal splits of the cryosections. The extent of splits caused by mAb LH7.2 was less pronounced than splits in sections incubated with patients' sera. This may be due to the fact that, in contrast to patients' autoantibodies, LH7.2 is directed to a single epitope on type VII collagen. Recently, it was reported that the blister-inducing capacity of mAbs to the ectodomain of type XVII collagen/BP180, a major target of autoantibodies in bullous pemphigoid patients, was enhanced when 2 mAbs were concurrently injected into human skin grafted onto athymic mice compared with the single use of these mAbs (Egan *et al,* 1999). These and our own results suggest that the extent of split formation may be increased when antibodies target different epitopes on the same autoantigen. In addition, mouse IgG1 may have a weaker

affinity for Fc receptors expressed on human neutrophils in comparison with human IgG. This may lead to less neutrophil adhesion to the DEJ and subsequently to a lesser dermal-epidermal separation by mouse monoclonal IgG1 antibody when compared with autoantibodies from EBA patients.

In a further set of experiments, we studied the importance of leukocytes for dermal-epidermal separation in this model. With shorter incubation times (30 to 90 minutes), recruitment of leukocytes to the DEJ predominated over the induction of a cleavage, whereas the extent of dermal-epidermal separation gradually increased with longer incubation and reached its maximum after an incubation of 2 h. By two independent lines of evidence we demonstrate that dermal-epidermal separation of the cryosections, induced by autoantibodies to type VII collagen, is dependent on the presence of leukocytes: i) omitting leukocytes abolished the induction of subepidermal splits; ii) in contrast to undigested antibodies, their $F(ab')_2$ fragments, that lacked the effector (Fc) portion, lost the blister-inducing ability when incubated with the cryosections. Leukocytes at the DEJ were activated, as demonstrated by their ability to generate a respiratory burst. In contrast to granulocytes, preparations of mononuclear cells were not able to mediate subepidermal cleavage.

Conclusion

This study demonstrates that antibodies to the NC1 domain of type VII collagen mediate an Fcγ-dependent neutrophil activation and induce dermal-epidermal separation in cryosections of human skin. Future studies will be aimed at characterising the factors that determine the blister-inducing potential of autoantibodies to type VII collagen, especially the pathogenically relevant IgG subclasses and their interaction with Fcγ receptors expressed on leukocytes. In addition, this experimental model may eventually contribute to the development of novel therapeutic strategies for this disease.

Summary

Epidermolysis bullosa acquisita (EBA) is an autoimmune subepidermal blistering disease associated with autoantibodies to type VII collagen, the major constituent of anchoring fibrils. Previous attempts to demonstrate the blister inducing potential of autoantibodies to this protein have failed. To address this question, we used an *in vitro* model involving cryosections of human skin incubated with patients' autoantibodies and leukocytes from healthy donors. We show that sera from 14 out of 16 EBA patients, in contrast to sera from healthy controls, induced dermal-epidermal separation in the cryosections. The level of the experimentally induced split localizes to the lamina lucida of the dermal-epidermal junction. Recruitment and activation of neutrophils at the dermal-epidermal junction was necessary for split induction, whereas mononuclear cells were not required. Importantly, patients' autoantibodies affinity-purified against a recombinant form of the non-collagenous 1 (NC1) domain of type VII collagen retained their blister-inducing capacity, while patients' IgG that was depleted of reactivity to type VII collagen lost this ability. Monoclonal antibody LH7.2 to the NC1 domain of type VII collagen also induced subepidermal splits in the cryosections; $F(ab')_2$ fragments of autoantibodies to type VII collagen were not pathogenic. These findings demonstrate the capacity of autoantibodies to type VII collagen to trigger an Fcγ-dependent inflammation leading to split formation in cryosections of human skin.

Zusammenfassung

Die Epidermolysis bullosa acquisita (EBA) ist eine subepidermal blasenbildende Autoimmundermatose, die mit Autoantikörpern gegen Typ VII Kollagen, den Hauptbestandteil der Verankerungsfibrillen der dermo-epidermalen Junktionszone (DEJ), assoziert ist. Bislang war jedoch unklar, ob diese Autoantikörper tatsächlich eine Blasenbildung verursachen. In der vorliegenden Arbeit gingen wir dieser Frage unter Verwendung eines Gefrierschnitt-Modells nach. Nach Koinkubation mit Leukozyten gesunder Spender induzierten 14 von 16 EBA-Seren eine subepidermale Spaltbildung, nicht jedoch die Seren von gesunden Freiwilligen. Die Spaltbildung erfolgte im Bereich der Lamina lucida der DEJ und war von der Rekrutierung und Aktivierung neutrophiler Granulozyten, nicht jedoch von der Präsenz mononuklearer Zellen abhängig. Autoantikörper von Patienten, die gegen eine rekombinante Form der NC1-Domäne des Typ VII Kollagens affinitätsaufgereinigt wurden, und der gegen die NC-1-Domäne gerichtete monoklonale Antikörper LH7.2 induzierten ebenfalls eine subepidermale Spaltildung. Dagegen führte die Präadsorption der EBA-Seren mit rekombinantem Typ VII Kollagen zum Verlust des blaseninduzierenden Potentials. Diese Fähigkeit verloren auch durch Pepsinverdau hergestellte F(ab')2-Fragmente der Patienten-Autoantikörper gegen Typ VII Kollagen. Die Ergebnisse dieser Arbeit zeigen, dass Autoantikörper gegen Typ VII Kollagen eine Fcγ-abhängige Entzündung und subepidermale Spaltbildung in Gefrierschnitten humaner Haut hervorrufen.

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