Characterization of basal membrane antigenic epitopes using specific antibodies

Summary of the doctoral thesis

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Cluj - Napoca
2012
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Mechanisms of autoantibody mediated blister formation in BP and EBA
Adhesion molecules in the skin
Autoimmune sub-epidermal blistering diseases

Granulocyte mediated tissue damage in subepidermal blistering diseases

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Abbreviations

ABD-actin binding domain
ADAM family- A Disintegrin And Metalloproteinase family
APC - antigen presenting cell
BMZ - basement membrane zone
BP - bullous pemphigoid
BP180 - bullous pemphigoid antigen of 180 kDa
BP230 - bullous pemphigoid antigen of 230 kDa
BSLE – systemic lupus erythematosus
CMV – Cytomegalovirus
CD – Crohn's disease
DEB - dystrophic epidermolysis bullosa
DEJ - dermal-epidermal junction
DH – dermatitis herpetiformis
DSC-desmocollin
DSG-desmoglein
EAE - Experimental Autoimmune Encephalomyelitis
EBA – epidermolysis bullosa acquisita
EBV- Epstein-Barr virus
EDTA – ethylenediamine tetraacetic acid
ELISA-Enzyme-linked immunosorbent assay
HHV - Human herpes virus
HIV- Human immunodeficiency virus
GST – glutathione S-transferase
HSV-Herpes simplex virus
IBD - inflammatory bowel disease
IF – immunofluorescence
IPTG – isopropyl β-D-thyogalactopyranoside
kDa– Kilodalton
LAD – linear IgA disease
LAD-1 – linear IgA disease antigen 1
LB – Luria-Bertani medium
MHC- major histocompatibility complex
MMP - mucous membrane pemphigoid
Key words

auto-antibodies
bullous pemphigoid
collagen VII
collagen XVII
ELISA
epidermolysis bullosa acquisita
neo-epitopes
reactive oxygen species
Introduction

Adhesion molecules in the skin

Tissue formation in multicellular organisms results from the capacity of cells to attach to other cells or non-cellular structures. Skin has a complex structure and its integrity is crucial for its barrier and protective function.

Adhesion molecules are the ones that provide the connection between epidermis and the subjacent layer namely the basement membrane. The importance of skin integrity is underlined by the genetic and autoimmune diseases associated with lack or loss of the adhesion molecules, respectively (Chan, 1997; Moll and Moll, 1998).

Desmosomes are the most common intercellular adhering junction in epithelial cells playing an important role in maintaining the proper structure of the skin. Epidermal cells are connected through the interaction of the intracellular plaque of the desmosome composed of desmoplakin and plakoglobin with cytokeratin filaments and transmembranous molecules such as desmogleins and desmocollins (Hertl et al., 2006; Delva et al., 2009).

Skin basement membrane (BM) is a connective structure between the epidermis and the dermis. As revealed by electronic microscopy, the BM has four distinct subregions: the cytoskeleton, hemidesmosomes and plasma membranes of basal keratinocytes, the lamina lucida, the lamina densa (or BM proper) and a sublamina densa region in the papillary dermis (Figure 1) (Olasz and Yancey, 2008).
Figure 1. The most common basal membrane proteins which were suggested to be autoantigens in autoimmune sub-epidermal blistering diseases. Basal keratinocytes are connected to the dermis through the hemidesmosomal adhesion complex. Intermediate filaments (keratin filaments) mediate cytoskeleton connections to the plasma membrane. BP 230 is an intracellular protein which interacts with keratin filaments and the intracellular domain of BP 180. BP 180 is a transmembrane protein with an essential role in dermal epidermal cohesion. Its role is suggested by the dermal-epidermal separation and blister formation in bulous pemphigoid patients which have auto-antibodies against this protein or in those with mutations in the COL17A1 gene. BP 180 is connected with plectin, laminin 5 and α6β4 integrin. Lamnin 5 is an adhesive ligand for the transmembrane α6β4 integrin. P 200, a novel autoantigen at the basement membrane is a 200 kDa protein localized within the lower lamina lucida.

Autoimmune sub-epidermal blistering diseases

Autoimmune blistering skin diseases are organ specific diseases associated with an immune response mounted against adhesion proteins promoting cell-cell or cell-matrix adhesion in the skin (Olasz and Yancey, 2008). Both autoreactive T cells and autoantibodies are encountered in patients with these diseases but clinical observations and experimental studies suggest that blister induction is mainly mediated by autoantibodies (Sitaru, 2007). Patients’ autoantibodies have been
used to identify target autoantigens and demonstrated that they represent important structural proteins in the epidermal basement membrane.

Based on clinical, histopathological, and immunopathological criteria, autoimmune bullous diseases are classified into four major groups: pemphigus diseases, pemphigoid diseases, epidermolysis bullosa acquisita, and dermatitis herpetiformis Duhring (Mihai and Sitaru, 2007a).

**Epidermolysis bullosa acquisita**

Epidermolysis bulosa acquisita (EBA) is a subepidermal blistering disease associated with antibodies directed to type VII collagen, the main component of anchoring fibrils at the dermal epidermal junction. EBA autoantibodies label the dermal side of 1 M NaCl split skin by indirect immunofluorescence (Woodley and Chen, 2004;Woodley et al., 1984;Gammon et al., 1984). As in the case of other autoimmune diseases, the aetiology of EBA is still unknown. However an association with the presence of HLA –DR alleles in EBA patients has been suggested (Gammon et al., 1988).

EBA is a rare disease of elderly although some juvenile cases were reported (Sitaru, 2007;Callot-Mellot et al., 1997).The first criteria for EBA diagnosis including negative family and personal history for bullous disease, adult onset of the eruption, spontaneous or trauma-induced blisters resembling those of hereditary dystrophic epidermolysis bullosa (DEB), exclusion of all other bullous diseases, were defined by Roegnik et al., four decades ago (Roenigk et al., 1971).

Like DEB, EBA is clinically characterized by skin fragility, blisters, and erosions over trauma-prone parts of the body, and lesions that heal with scarring and milia formation (Woodley and Chen, 2004). According to clinical manifestations, five variants of EBA have been described: (1) a classical presentation; (2) a bullous pemphigoid (BP)-like presentation; (3) a cicatricial pemphigoid (CP)-like presentation; (4) a presentation reminiscent of Brunsting-Perry pemphigoid with scarring lesions predominantly localized to the head and neck; (5) a presentation similar to linear IgA bullous dermatosis (LABD) or chronic bullous disease of the childhood (Remington et al., 2008).

Type VII collagen is a homotrimer protein with a helical structure consisting of 3 identical α1 polypeptide chains. Each of the chains consists of a - 145 kD central collagenous domain characterized by a repeating Gly-X-Y amino acid sequence which is interrupted by several imperfections. The collagenous domain is flanked at the amino-terminal end by a large, - 145 kD
noncollagenous domain, NC-1, and by a smaller, -20 kD noncollagenous domain, NC-2, at the carboxy terminal end (Uitto et al., 1992; Christiano et al., 1992). In the extracellular space, a portion of the NC-2 domain is proteolytically removed (Bruckner-Tuderman et al., 1995).

Epitope mapping studies have shown that the main antigenic epitopes are localized within the NC1 domain of collagen VII (Lapiere et al., 1993). In 2001, Chen et al., described a novel antigenic epitope at the level of carboxyl terminal domain of collagen VII (Chen et al., 2001a).

The genetic susceptibility associated with HLA-DR alleles suggests that T cells can play a role in the initiation of the disease. Experimental observations clearly demonstrated that EBA is a typical autoantibody mediated diseases. The pathogenicity of autoantibodies specific to collagen VII was first demonstrated using an ex vivo model (Sitaru et al., 2002a). In this model, autoantibodies were shown to induce dermal-epidermal separation when co-incubated with normal human skin cryosections and human leukocytes from normal donors. In this model, leukocytes recruitment and activation at the dermal epidermal junction are dependent on the presence of the Fc portion of the antibodies.

It has been shown that autoantibodies generated against murine or human type VII collagen in rabbits or isolated from EBA patients induced blister formation when passively transferred into mice (Sitaru et al., 2005; Woodley et al., 2006; Woodley et al., 2005). Moreover, immunization of susceptible mice strains with the recombinant form of murine type VII collagen resulted in blistering diseases reproducing clinical, histopathological, and immunopathological findings in EBA patients (Sitaru et al., 2006). Recently, using this animal model, Sitaru et al, showed that T cells are required to induce EBA in mice immunized with collagen autologous VII (Sitaru et al., 2010).

The laboratory diagnosis of EBA relies on several laboratory tests, including detection of tissue-bound autoantibodies by direct IF microscopy and demonstration of serum autoantibodies binding to the dermal side of the 1M salt-split skin by indirect IF microscopy. The definitive diagnosis of EBA requires characterization of the molecular specificity of autoantibodies (Mihai and Sitaru, 2007a). Autoantibodies against collagen VII may be detected by immunoblotting and/or ELISA using recombinant proteins (Mihai and Sitaru, 2007a). For detection of collagen VII-specific autoantibodies by ELISA, immunoassays using recombinant forms of the NC1 domain or the full-length molecule have been developed (Chen et al., 1997; Pendaries et al., 2010; Saleh et al., 2011).
**Bullous pemphigoid (BP)**

BP is a chronic subepidermal blistering disease usually seen in the elderly although a few childhood cases were reported. Circulating and tissue bound antibodies against two hemidesmosomal proteins: BP230 and BP 180 can be detected in BP patients (Yancey, 2005).

BP 230 also named BP antigen 1 was identified as BP autoantigen by Stanley et al., in 1981 (Stanley et al., 1981). By isolation of the corresponding cDNA and cloning, Sawamura et al. have shown that the BP 230 gene maps the short arm of the chromosome six (Sawamura et al., 1990).

BP 230 is an intracellular protein localized at the level of the hemidesmosomal plaque mediating the attachment of the keratin intermediate filaments to the hemidesmosomal plaque (Borradori and Sonnenberg, 1999). It has been shown that BP 230 recruitment to hemidesmosome depends on the interaction of this protein with BP 180 and β4 integrin. In the keratinocytes which lack BP 180, BP 230 cannot be localized properly into hemidesmosome even if β4 integrin and plectin are present suggesting that these two proteins are not sufficient for proper recruitment of P 230 (Borradori et al., 1998; Koster et al., 2003). The biological function of this protein in the organization of cytoskeletal intermediate filaments in basal keratinocytes is supported by the fact that BPAG1 knockout mice show signs of epithelial fragility (Guo et al., 1995). BP 230 has also been reported to play a pathogenic role in BP as suggested by the ability of rabbit IgG directed to an antigenic fragment of the murine BP230 to induce blisters in mice (Kiss et al., 2005). However, our recent data challenge this hypothesis and show that BP230 specific antibodies have rather limited immunopathologic properties in experimental BP (Feldrihan et al., unpublished observations).

BP 180 is a transmembrane protein of the hemidesmosome anchoring filaments which links the basal keratinocytes to the basement membrane. It is a homotrimer consisting of three indentical α1 chains with intracellular localized NH$_2$ terminus, a short transmembrane fragment and an extracellular COOH domain. The ectodomain consists of 15 collagenous subdomains (COL1–COL15), characterized by typical collagenous GXY repeat sequences flanked by 16 short non-collagenous sequences (NC1–NC16A) (Franzke et al., 2003). The ectodomain of collagen XVII is constitutively shed from the cell surface in vitro (Franzke et al., 2009; Hirako et al., 1998; Schlacke et al., 1998) by proteolytic enzymes from ADAM (A Disintegrin And Metalloproteinase) family (Franzke et al., 2009).

Although the presence of autoantibodies against both BP230 and BP180 is a characteristic feature of BP patients, the role of BP 230 in pathogenesis is still not clear. As previously mentioned,
subepidermal blisters were induced by injection of neonatal mice with rabbit antibodies generated against BP230 (Kiss et al., 2005) results that are not supported by our recent studies. In contrast, the pathogenicity of BP 180 autoantibodies is supported by several experimental models. The first evidence that BP autoantibodies are pathogenic comes from an ex vivo model developed by Gammon and coworkers (Gammon et al., 1981; Gammon et al., 1980). In this model the authors incubated normal human skin cryosections with BP sera and subsequently added leukocytes isolated from the blood of healthy volunteers. They demonstrated that recruitment of leukocytes at the dermal-epidermal junction depends on the complement activation.

In another set of experiments they showed that the leukocytes attached at the basal membrane of cryosections incubated with BP patients' serum were subsequently activated and induced dermal-epidermal separation (Gammon et al., 1982). More recently, using the same experimental model, Sitaru et al. have reported that BP sera immunoabsorption against the recombinant NC16A domain of BP180 abolished the capacity of BP patients' sera to induce dermal-epidermal separation. In contrast, IgG autoantibodies affinity-purified against recombinant BP180 from BP patient’s sera induced dermal–epidermal separation in this model suggesting the pathogenicity of NC16A specific BP180 autoantibodies (Sitaru et al., 2002b).

In 1993, Liu et al., demonstrated the pathogenicity of BP 180 autoantibodies in vivo. Passive transfer of rabbit IgG developed against the murine collagen XVII into neonatal mice induced clinical, histologic, and immunopathologic alterations similar to those observed in patients with BP (Liu et al., 1993). Using this model, it has been shown that activation of the classical pathway of complement, neutrophils, mast cells and macrophages are essential for the experimental induction of BP (Chen et al., 2001b; Chen et al., 2002a; Nelson et al., 2006; Liu et al., 2000; Liu et al., 1995). Attempts to induce a BP-like phenotype in mice by passive transfer of BP patients autoantibodies have failed. It was suggested that this failure is likely due to differences between human and murine collagen XVII aminoacid sequence (Anhalt and Diaz, 1987). To overcome this impediment Olasz et al generated a transgenic mouse expressing human collagen XVII under keratin 14 promotor control. Wild type mice grafted with skin from these transgenic mice developed IgG antibodies against human collagen XVII associated with an inflammatory immune response consisting of neutrophil infiltration, IgG and C3 depositions at basement membrane of grafted skin leading to BP like subepidermal blister formation (Olasz et al., 2007). Neonatal human collagen XVII transgenic mice injected with IgG from BP patients showed epidermal detachment by gentle friction of the skin at 48hrs after injection. This mouse model reproduced the PB phenotype and demonstrated for the first time the in vivo pathogenicity of PB patients.
autoantibodies (Nishie et al., 2007).

**Aims of the study**

Despite important advances in our understanding of disease pathogenesis, BP has remained a life-threatening disease. Largely, the body of knowledge that we now have about BP comes from the modeling of the disease in neo-natal mice. Currently accepted models are based on the passive transfer of rabbit antibodies into neo-natal wild-type mice or more recently patients' antibodies into genetically modified mice. The main drawbacks of the existing models are represented by the fact that the disease is not occurring spontaneously and that the observation period is limited to a couple of days thus precluding any accurate evaluation of the time course of the pathogenesis and possible therapeutic approaches. Therefore the first aim of the present study was to:

1. Obtain and characterize polyclonal antibodies produced by immunizing rabbit and sheep with different fragments of type XVII collagen epitopes, antibodies that could be used to transfer BP into adult mice.

2. Study the pathogenesis of antibodies specific to a collagen XVII neo-epitope using an *ex vivo* model of bullous pemphigoid.

3. Investigate the effect of exogenous bovine superoxide dismutase (SOD) and myeloperoxidase (MPO) inhibition in the *ex vivo* DES induced by bullous pemphigoid pathogenic antibodies.

4. Develop a novel sensitive ELISA assay for detecting collagen specific antibodies in patients with EBA and inflammatory bowel disease, respectively.
Materials and methods

Human sera

Serum samples were obtained from patients with EBA (n=50), Crohn's disease (CD; n=50), ulcerative colitis (UC; n=50), bullous pemphigoid (BP; n=76), and pemphigus vulgaris (PV; n=42) before initiation of treatment and healthy donors (n=245). The study was approved by the Ethics Committee of the Medical Faculty of the University of Freiburg, Germany (Institutional Board Projects no 318/07, 425/08 and 278/11). We obtained informed consent from patients whose material was used in the study, in adherence to the Helsinki Principles.

Isolation of peripheral blood cells from healthy donors

Leukocytes were isolated by dextran sedimentation. Blood obtained from healthy donors was mixed 1:1 with 3% dextran (Roth) prepared in 0.9% NaCl solution, in 50 ml falcon tubes. After 30 minutes most of the erythrocytes sedimented at the bottom of the tube and the supernatant was collected and the cells were pelleted by centrifugation. To eliminate the remaining erythrocytes, the pellet was resuspended with 20ml of NaCl 0.2% solution. After 30 s the hypothonic lysis was stopped by adding a volume of 20ml of 1.6% NaCl solution. Cells were pelleted by centrifugation at 1200rpm for 7min at room temperature, washed with 20 ml DMEM medium (Lonza) and resuspended in an appropriate volume of DMEM to obtain the density of 3x10^7 cells/ml. The viability of the cells was tested with trypan blue and cell suspensions with viability >95% were used in the study. Cells were kept on ice until used.

Ex vivo induction of dermal-epidermal separation by pathogenic antibodies

To test the pathogenicity of rabbit antibodies against recombinant forms of human collagen XVII, we used the ex vivo model of bullous pemphgoid as reported by Sitaru et al. (Sitaru et al., 2002b). Briefly, BP rabbit sera were incubated for 3 hours with human skin cryosections in a humid chamber. After being washed with PBS the sections were incubated with 300µl of leukocytes suspension for another 3 hours at 37°C in a humidified incubator. At the end of this interval, skin
section were washed twice with PBS, fixed in formalin and stained with hematoxylin and eosin.

**Generation of the recombinant forms of human collagen VII**

cDNA sequences corresponding to non-collagenous domains of human collagen VII (NC1, NC2) were obtained by PCR amplification on 8xHis tagged full length collagen VII sequence (kind gift from A Fritsch), previously cloned into prokaryotic vector pcDNA3.1 Zeo(-), Invitrogen (Fritsch et al., 2009). Primers for polymerase chain reactions (PCR) were synthesized by Eurofins MWG (Ebersberg, Germany; Table 1). Restriction sites for EcoRI and HindIII were introduced by primers. Briefly, pcDNA3.1hc7 vector containing the full length sequence of human collagen VII was digested with EcoRI and AgeI restriction enzymes. The digested vector containing the sequence spanning aminoacids 1-443 was ligated with the PCR fragment overlapping the restriction site for AgeI within the collagen VII sequence resulting in the recombinant vector pcDNA3.1hCol7NC1 containing the entire sequence of NC1 domain of collagen VII spanning the aminoacids 1-1278. The PCR product corresponding to the NC2 fragment was digested with EcoRI and HindIII restriction enzymes and ligated into pcDNA3.1hCol7NC1 vector digested with the same enzymes resulting in the recombinant vector pcDNA3.1hCol7NC1-NC2 with the sequence spanning the aminoacids 1-1278 and 2776-2944. Subsequently, the recombinant fragment (NC1-NC2) was subcloned into pcDNA5FRT vector with CMV promoter using NheI and HindIII restriction enzymes resulting in the pcDNA5FRTcol7NC1-NC2 recombinant vector. To obtain the recombinant protein containing NC1, hinge region and NC2 domains of type VII collagen the nucleotide sequence coding the hinge region and NC2 domain, flanked by the restriction sites for EcoRI and Hind III, was synthesized by GenScript in pUC57 vector. Further, the sequence was cut out with EcoRI and HindIII restriction enzymes and ligated into the pcDNA5FRT NC1-NC2 vector digested with the same enzymes resulting in the pcDNA5FRTcol7NC1-H-NC2 recombinant vector containing the sequence spanning the aminoacids 1-1278, 1940-1979 and 2776-2944. Correct DNA sequences of all vectors were confirmed by direct sequencing.

**Expression of recombinant collagen VII fragments in Hek293 mammalian cells**

Flp-in Hek293 T host cells were transfected with 5µg of pcDNA5FRTcol7NC1-NC2/pcDNA5FRTcol7NC1-H-NC2 and 2.5µg of pOG44 vectors in lipofectamine2000 (Invitrogen).
Transfected cells expressing the desired proteins were selected under 200µg/ml hygromicine (Roth). Proteins were precipitated from culture medium with 50% ammonium sulphate for 4 hours at 4°C and collected by centrifugation at 27200 rcf for 45 minutes at 4°C. The proteins were resuspended in cold PBS, dyaalised overnight against PBS and purified by metallochelate affinity chromatography using nickel nitrilotriacetic acid coupled with agarose (Ni-NTA, Qiagen, Germany). Purified proteins were separated by SDS PAGE on 8% gels under reducing conditions and transferred on nitrocellulose membrane. Membrane strips were incubated with 1000-fold diluted monoclonal antibody specific for human collagen VII (clone LH 7.2; Chemicon International, Germany) and reactivity was detected with secondary, HRP-conjugated goat anti-mouse IgG antibodies (Abcam).

**Statistical analysis**

A ROC curve allows for exploring the relationship between the sensitivity and specificity of the ELISA for a variety of different cut-off points, thus allowing the determination of an optimal cut-off point for positivity. Therefore, to determine the cut-off value for the ELISA using recombinant forms of type VII collagen, we performed a ROC analysis by plotting on the X-axis the 1 - specificity (the false positive rate) and on the Y-axis the sensitivity (the true positive rate). The coefficient of variation (CV) was calculated as follows: CV= %CV= (standard deviation/mean) x 100. Statistical analyses were performed using the GraphPad Prism statistical package (v5; GraphPad Software, San Diego, CA). Statistical significance was calculated using the non-parametric Mann-Whitney-U test and correlations were analyzed by the Spearman's rank correlation test; p <0.05 was considered significant.
Results

Rabbit and sheep antibodies to type XVII collagen bound at the dermal-epidermal junction of murine skin and fixed the murine complement

Circulating antibodies obtained at different time points from animals immunized with recombinant fragments of murine collagen XVII were tested for their ability to recognize the protein in situ. Antibodies from both rabbit and sheep showed a linear staining of the basal membrane by IF microscopy using murine skin as a substrate (Figure 2a, 2b). In contrast, antibodies obtained before the first immunization did not bind to the DEJ (Figure 2c). When incubated with 1M NaCl–split mouse skin, antibodies from immunized animals stained the epidermal side of the substrate (Figure 2d and 2e). Antibodies purified from preimmune sera did not bind to the skin (Figure 2f). Additionally, sera from immune (Figure 2g and 2h) but not preimmune animals (Figure 2i) elicited deposition of murine C3 at the DEJ, as shown by the complement fixation test using murine skin as a substrate.

Figure 2. Antibodies from immunized animals recognize mouse type XVII collagen and activate complement ex vivo. Serum was obtained from animals before and after the immunization.
at different time points. Immune rabbit (a) and sheep (b) antibodies bound to the basal membrane of the mouse skin. In contrast, antibodies obtained from a rabbit before the first immunization (c) did not recognize the antigen in situ. When incubated with mouse split skin, immune antibodies from both the rabbit (d) and sheep (e) bound to the epidermal side of the artificial blister in contrast to antibodies from pre-immune sera which didn't stain the skin (f). (magnifications 200x). Immune antibodies from the rabbit (g) and sheep (h) activated murine complement when incubated on cryosections of murine skin. Both rabbit and sheep (i) primmune antibodies didn't fix the complement (magnifications 200x).

Antibodies to type XVII collagen recognize recombinant forms of the protein by western blotting

Recombinant proteins used to immunize the animals were separated by SDS page and then transferred to a nitrocellulose membrane. By immunoblot analysis, antibodies from immune sera, in contrast to pre-immune or normal sera, recognized recombinant forms of collagen XVII (Figure 3). The main reactivity could be detected against the mCXVII-EC1 fragment (shown in line 2).
Antibodies specific to a collagen XVII neo-epitope fixed complement and induced dermal – epidermal separation *ex vivo*

A major diagnostic and pathogenic feature of pemphigoid IgG autoantibodies is represented by their capacity to activate the complement system (Sitaru et al., 2002b). To assess the capacity of rabbit IgG Abs recognizing different forms of collagen XVII to bind complement, an *in vitro* complement-fixation assay was performed using rabbit antibodies generated against the NC16A (p-Ab-NC16A) domain of human collagen XVII and Ab 83457, a collagen XVII neoepitope-specific antibody. Both antibodies bound to the dermal-epidermal junction of normal human skin and showed complement-fixing activity (*Figure 4a, 4b, 4d, 4e*). In contrast, the IgG fraction from a preimmune rabbit did not stain the dermal-epidermal junction and did not show complement-binding capacity (*Figure 4c, 4f*). Several lines of evidence indicate that recruitment and activation of granulocytes by autoantibodies after binding to the dermal-epidermal junction are a prerequisite for blister formation in pemphigoid diseases (Sitaru et al., 2002b; Sitaru et al., 2004). Therefore, to characterize the granulocyte-activating ability of the collagen XVII neoepitope-specific antibody, we used an *ex vivo* antibody-induced granulocyte-dependent assay having as readout dermal-epidermal splits in cryosections of normal human skin. Incubation of cryosections with the pAb-NC16A (*Figure 4g*) and Ab 83457 (*Figure 4h*) but not with the control IgG (*Figure 4i*), resulted in subepidermal cleavage when co-incubated with leukocytes from healthy donors.
Figure 4. Neoepitope-specific Ab 83457 against the human collagen XVII ectodomain fixes complement and induces dermal-epidermal separation on human skin cryosections. IgG from rabbits immunized against the entire NC16A domain (pAb-NC16A), Ab 83457, and preimmune rabbit serum was incubated with human skin cryosections. IgG from pAb-NC16A (a) and Ab 83457 (b), but not preimmune serum (c) bound to the dermal-epidermal junction. When fresh human serum as source of complement was added, both pAb-NC16A (d) and Ab 83457 (e) fixed complement C3 at the dermal-epidermal junction. In contrast, preimmune serum (f) did not fix complement. Subsequent incubation with normal human leukocytes resulted in dermal-epidermal separation in sections treated with IgG from pAb-NC16A (g) and Ab 83457 (h), but not from preimmune serum (i). Original magnification x400
Generation and purification of the recombinant forms of human collagen VII (hCVIINC1-NC2, hCVIINC1-H-NC2)

The recombinant proteins were expressed in mammalian cells and purified by metallochelate affinity chromatography. When separated by SDS-PAGE, the recombinant collagen VII forms containing the NC1 fused with the NC2 domain (His-hCVII-NC1-NC2) as well as the hinge region (His-hCVII-NC1-H-NC2), migrated consistently with their calculated molecular masses of 153 kDa (Figure 5b, lane 2) and 158 kDa (Figure 5b, lane 3), respectively. A monoclonal antibody specific for the NC1 domain of collagen VII recognized both recombinant forms by immunoblot analysis (Figure 5c, lanes 1 and 2).
Figure 5. Recombinant forms of collagen VII used in this study. (a) Schematic representation of human collagen VII. Collagen VII consists of a central collagensous domain flanked by a large, 145 kDa N-terminal non-collagensous domain and a smaller, 30 kDa non-collagens domain at its C-terminus. The collagensous domain is interrupted by a 39 amino acid non-collagensous hinge region. The recombinant forms of collagen VII generated in this study are two N-terminally 8xhistidine tagged chimeric proteins termed His-hCVII-NC1-NC2 and His-hCVII-NC1-H-NC2 corresponding to the fused NC1 and NC2 domains (aa 1-1278, 2776-2944) and to the fused NC1, hinge and NC2 regions (1-1278, 1940-1979, 2776-2944) of the antigen, respectively. (b) Sodium dodecylsulfate-polyacrylamide gel electrophoresis of the purified recombinant His-hCVII-NC1-NC2 and His-hCVII-NC1-H-NC2 proteins shows their migration at around 155 (lane 2) and 160
kDa (lane 3), respectively. Weight markers of 250, 150, 100, 75 and 50 kDa are shown in lane 1. (c) Immunoblot analysis of the two recombinant proteins His-hCVII-NC1-NC2 (lane 2) and His-hCVII-NC1-H-NC2 (lane 3) using a monoclonal antibody specific to the NC1 domain of collagen VII (clone LH7.2).

**EBA autoantibodies reacted with both recombinant forms of collagen VII**

The immunoreactivity of the newly expressed recombinant chimeric forms of collagen VII was analyzed by immunoblotting using sera from reference EBA patients and healthy donors. Representative examples are shown in Figure 6. IgG autoantibodies from EBA patients' sera (n=5) recognized the recombinant forms His-hCVII-NC1-NC2 (Figure 6, lanes 1-3) and His-hCVII-NC1-H-NC2 (Figure 6, lanes 5-7) of collagen VII. None of the normal human sera (n=2) reacted with these recombinant forms of collagen VII (Figure 6, lane 4 and 8).

![Figure 6](image)

**Figure 6.** Immunoreactivity of EBA autoantibodies with the recombinant forms of collagen VII. Purified, recombinant His-hCVII-NC1-NC2 (lanes 1-4) and His-hCVII-NC1-H-NC2 (lanes 5-8) were electrophoretically separated by 8% SDS-PAGE, transferred to nitrocellulose and
immunoblotted with EBA patient's sera (lanes 1-3 and 5-7) and normal human sera (NHS) (lanes 4 and 8).

Development of the ELISA’s using recombinant collagen VII NC1-NC2

To develop a novel ELISA test for specific detection of antibodies against collagen VII in patient’s sera we used HEK293 mammalian cells to produce a recombinant protein comprising both non-collagenous domains of human collagen VII. To establish the working conditions, including antigen amount/well, dilution of sera and secondary antibodies an initial chessboard titration was performed (data not shown). To determine the cut-off value of the newly established immunoassay, we performed a ROC analysis of the ELISA readings with sera from 50 EBA and 160 healthy donors. The cut-off value of the newly immunoassay was set at 0.42 corrected OD reading units with a calculated sensitivity of 92% and specificity of 97.5% (Figure 7).
Figure 7. Receiver-operating-characteristic (ROC) curve. AUC, area under the curve. The test was performed with sera from patients with epidermolysis bullosa acquisita (n=50) and healthy donors NHS=160).
Recent studies have shown that there are some EBA sera which reacted with the triple helical collagenous domain of collagen VII. Based on these observations, we developed another ELISA using a recombinant protein which contains besides the two non-collagenous domains of collagen VII the hinge region which is a non-collagenous fragment interrupting the large collagenous domain consisting of 39 aminoacids. This immunoassay was subsequently used to characterize the prevalence of collagen VII-specific autoantibodies and their IgG subclass in large cohorts of patients with IBD, pemphigus and pemphigoid diseases as well as healthy donors. ROC analysis of the ELISA readings with same EBA sera and controls used for NC1-NC2 ELISA allowed us to set the cut-off value of the newly established immunoassay. Based on a calculated specificity of 97.5% and a sensitivity of 94% the cut-off was set at 0.32 corrected OD reading units (Figure 8).
Figure 8. Receiver-operating-characteristic (ROC) curve. Test performed with sera from patients with epidermolysis bullosa acquisita and controls.
ELISA using recombinant NC1-NC2 and NC1-hinge-NC2 forms of collagen VII allow for sensitive and specific detection of antigen-specific autoantibodies

Applying the cut-off value of 0.322 defined by ROC analysis for the newly developed ELISA showed that 47 EBA (94%; 95% CI: 87%-100%; n=50), 2 CD (4%; 95% CI: 0%-9.43%; n=50), 8 UC (16%; 95% CI: 5.8%-26%; n=50), 2 BP (2.63%; 95% CI: 0%-6.23%; n=76), 4 PV (9.52%; 95% CI: 0%-18.4%; n=42) patients and 4 of the healthy donors (1.63%; 95% CI: 0%-3.21%; n=245) showed IgG reactivity against the chimeric NC1-hinge-NC2-hCVII protein (Figure 9; Table 4). Therefore, a sensitivity and a specificity of 94% (95% CI: 83.4%-98.75%) and 97.50% (95% CI: 94%-100%), respectively, were calculated for the ELISA detecting collagen VII-specific IgG autoantibodies in patients with EBA. The area under the curve (AUC) was 0.984 (95% CI: 96.3%-100%) indicating an excellent discriminatory power.
Figure 9. ELISA reactivity of epidermolysis bullosa acquisita (EBA) and control sera with the recombinant NC1-H-NC2 noncollagenous domains of collagen VII. Scatter plots represent optical density measurements of serum reactivity of EBA, BP, PV, UC, CD patients and healthy donors with the purified recombinant His-hCVII-NC1-H-NC2 protein. The cut-off of the assay is represented by a dotted line.
Table 4. Sensitivity and specificity of the ELISA with recombinant chimeric collagen VII.

<table>
<thead>
<tr>
<th>Sera</th>
<th>Positive/Total</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBA</td>
<td>47/50 (94%)</td>
<td>94% (87%-100%)</td>
<td>98.4% (94.90%-100%)</td>
</tr>
<tr>
<td>BP</td>
<td>2/76 (2.63%)</td>
<td>2.63% (0.0%-6.23%)</td>
<td>98.4% (94.90%-100%)</td>
</tr>
<tr>
<td>PV</td>
<td>4/42 (9.52%)</td>
<td>9.52% (0.0%-18.4%)</td>
<td>98.4% (94.90%-100%)</td>
</tr>
<tr>
<td>CD</td>
<td>2/50 (4%)</td>
<td>4% (0.0%-9.4%)</td>
<td>98.4% (94.90%-100%)</td>
</tr>
<tr>
<td>UC</td>
<td>8/50 (16%)</td>
<td>16% (5.8%-26%)</td>
<td>98.4% (94.90%-100%)</td>
</tr>
<tr>
<td>NHS</td>
<td>4/245 (1.63%)</td>
<td>1.63% (0.0%-3.21%)</td>
<td>98.4% (94.90%-100%)</td>
</tr>
</tbody>
</table>

The accuracy of the ELISA using only the NC1-NC2 domains of collagen VII was only slightly lower as demonstrated by a sensitivity of 92% (95% CI: 80.7%-97.7%) and a specificity of 97.50% (95% CI: 93.7%-99.3%) with an AUC of 0.980 (data not shown). The immunoassays using the 2 recombinant forms of collagen VII correlated well regarding their capacity to detect specific autoantibodies (r=0.95; p<0.0001).
Figure 10. ELISA reactivity of epidermolysis bullosa acquisita (EBA) and control sera with the recombinant NC1-NC2 noncollagenous domains of collagen VII. Scatter plots represent optical density measurements of serum reactivity of EBA, bullous pemphigoid (BP) patients and healthy donors with the purified recombinant His-hCVII-NC1-NC2. The cut-off of the assay is represented by a dotted line.
IgG levels by hCVII ELISA correlate with the IgG reactivity against the dermal-epidermal junction by IF microscopy

The indirect IF microscopy on salt-split skin is a standard diagnostic and monitoring tool in autoimmune bullous diseases. To further characterize the suitability of the newly developed ELISA for diagnosis of diseases associated with autoimmunity against collagen VII, we correlated the IgG levels by ELISA with the end-point titers by IF microscopy on salt-split skin in sera from patients with EBA (n=9) (Figure 11). When the IgG levels of collagen VII-specific IgG autoantibodies were plotted against the indirect IF microscopy titers, a positive correlation ($r=0.73$, $p<0.05$) was obtained. Interestingly, all sera from patients with BP (n=2), PV (n=4), CD (n=2) and UC (n=8) as well as from healthy donors (n=4), which showed low levels of IgG autoantibodies against collagen VII by ELISA, did not show binding to the dermal side by indirect IF microscopy on salt-split skin.

Figure 11 Indirect immunofluorescence on salt split skin. a. ELISA positive EBA serum recognized the antigen at the dermal side of the human salt split skin. b. an EBA serum positive by ELISA did not recognized the antigen by indirect immunofluorescence to determine the end point
Levels of autoantibodies against collagen VII do not correlate with inflammation markers in inflammatory bowel disease

C-reactive protein (CRP) is routinely used as marker of disease activity in patients with inflammatory bowel diseases, especially in CD. To address a possible direct role of collagen VII-specific autoantibodies in pathogenesis of inflammatory bowel disease, the ELISA levels of autoantibodies were correlated with the CRP values of the patients at the time of blood collection. The calculated correlation coefficients were $r=0.135$ (p=0.356) and $r=-0.174$ (p=0.231) for UC and CD patients, respectively.
**Discussion**

The ability of the immune system to discriminate between self and non-self antigens is crucial for the defense against the pathogenic microorganisms. When the immune system fails to tolerate self antigens an autoimmune response occurs. The causes and triggering mechanisms of the autoimmune reaction are still poorly understood.

Autoimmune diseases are in general rare disease but they are chronic, life-threatening and difficult to cure. Despite significant advances in our understanding of the disease pathogenesis, the current therapy is still based on systemic corticosteroids and/or other immunosuppressive agents which are less effective partly due to their numerous side effects.

For several of the autoimmune conditions *ex vivo* or/and *in vivo* experimental models have been developed and they have essentially contributed to a better understanding of the disease pathogenesis and in the future they will be very useful tools for dissecting the autoimmune phenomenon and for developing novel therapeutic strategies.

The most direct approach for reproducing autoantibody-induced autoimmune diseases *in vivo* has been to inject patients’ serum or purified antibodies specific to culprit autoantigens into healthy individuals. The method has been used for the first time by Harrington and coworkers in the early 1950s. They transferred sera from idiopathic thrombocytopenic purpura patients into themselves as healthy recipients (Harrington et al., 1951) reproducing signs of disease. Since then, several other autoimmune conditions, including myasthenia gravis (Naito et al., 1984), pemphigus vulgaris (Anhalt et al., 1982) and pemphigus foliaceus (Roscoe et al., 1985) have been successfully reproduced in experimental animals by transferring pathogenic antibodies from diseased individuals.

Previous attempts to reproduce BP by this “classical” transfer of the disease through antibodies from patients into experimental animals were unsuccessful (Anhalt and Diaz, 1987; Anhalt et al., 1981; Sams and Gleich, 1971;Gammon et al., 2002). This failure has been explained by a decreased level of cross-reactivity between human antibodies to type XVII collagen and the mouse skin due to the low degree of homology between the human and mouse type XVII collagen (Liu et al., 1993; Nishie et al., 2007; Nishie et al., 2009; Yamamoto et al., 2002; Liu et al., 2008). A further reason for the non-pathogenicity of pemphigoid patients autoantibodies in mice is related to their significantly weaker capacity of activating mouse innate immune factors when compared to human complement and granulocytes (Sesarman *et. al., submitted*) Therefore, in the
present study we have generated specific antibodies in rabbits and sheep which can be subsequently transferred into mice. This strategy used for the first time by Liu and co-workers to induce BP in neonatal mice (Liu et al., 1993) has been ever since used successfully for developing in vivo models for several other autoimmune diseases such as pemphigus vulgaris (Memar et al., 1996), anti-epiligrin cicatricial pemphigoid (Lazarova et al., 1996), and epidermolysis bullosa acquisita (Sitaru et al., 2005). Sheep immunization has proved to be a good alternative for rabbit. We have obtained relatively high volumes of serum per bleeding and the immune sera showed high titers by indirect IF microscopy on murine skin sections. Since the serum yield of a single sheep can be equivalent to that of eight to ten rabbits, using antibodies generated in sheep may offer an alternative for rabbit IgG.

According to their higher titers in the IIF and the more potent capacity to activate the complement system, sheep antibodies were shown to induce more rapid and severe disease when passively transferred into mice (Chiriac et. al., submitted). These recent results of our group could facilitate the work on elucidating the time course of the disease and hence could be useful in designing novel therapeutic strategies.

BP 180 is a transmembrane protein of the hemidesmosome anchoring filaments consisting of three identical α1 chains with NH2 terminus localized intracellular, a short transmembranous fragment and an extracellular COOH domain. The ectodomain of collagen XVII is constitutively shed from the cell surface in vitro (Franzke et al., 2009; Hirako et al., 1998; Schaecke et al., 1998) by proteases from ADAM family yielding a 120KDa extracellular protein known as Linear IgA disease antigen (LAD)-1 (Franzke et al., 2009). Post-translational cleavage of proteins can create neoepitopes, newly appeared antigenic sites on cleaved protein fragments, which are absent in the native precursors (Mort and Buttle, 1999). The fact that in BP and LAD, IgG or IgA class autoantibodies preferentially react with the shed ectodomain of collagen XVII (Marinkovich et al., 1996; Zone et al., 1998; Schumann et al., 2000) indicates that shedding of collagen XVII generates neoepitopes within the shed ectodomain, which may play a role in the pathogenesis of autoimmune blistering diseases. ADAMs do not require a specific cleavage consensus sequence, but cleave their transmembrane substrates at a defined length from the cell surface (Franzke et al., 2004; Zhao et al., 2001). Thus, it is highly likely that the main collagen XVII cleavage sites lie within the region Leu524-Gly526.

Another aim of the present study was to study the pathogenicity of an antibody (Nishie et al., 2010) generated against a possible neoepitope in the shed ectodomain of collagen XVII by testing its
ability to induce dermal-epidermal separation in vitro. Autoantibodies from patients with BP or epidermolysis bullosa acquisita show complement- and leukocyte-activating capacity, which appear to be main determinants of their pathogenicity (Shimanovich et al., 2004; Sitaru et al., 2002b). Therefore, we assessed these two major pathogenic features of the neoepitope-specific Ab 83457. Complement activation was measured by an immunofluorescence microscopy test assessing the classical pathway. The granulocyte-activating capacity of the antibody was evaluated by an ex vivo model of antibody-induced granulocyte-dependent dermal-epidermal separation in cryosections of human skin incubated with the antibody in the presence of leukocytes. These experiments demonstrated that, in analogy to human autoantibodies in BP, the neoepitope-specific antibody 83457 can fix human complement and induce granulocyte activation ex vivo.

In the last decades an important number of studies have focused on the role of protein post-translational modifications (PTMs) in autoimmune diseases. Several mechanisms by which proteins PTMs can trigger an autoimmune response have been postulated. Spontaneous or mediated by specific enzymes, post-translational modification of antigens can generate new MHC-peptide complexes which can activate T cells with high affinity T-cell receptor. This is the case of collagen type II, autoantigen in rheumatoid arthritis. In collagen induced arthritis, it has been proved that glycosylated collagen II is more arthritogenic than the non-glycosylated form (Michaëlsson et al., 1994).

Proteolytic cleavage of the antigens can generate neo-epitopes or can expose cryptic epitopes which can be recognized as non-self. In a recent study, Casciola-Rosen et al., have shown that granzyme B cleaves the ε subunit of the acetylcholine receptor in a site which corresponds with the epitopes recognized by the T cells in myastenia gravis and which have been reported as being the immunodominant suggesting that this event may be involved in the initiation of the autoimmune response (Casciola-Rosen et al., 2008).

Collagen XVII ectodomain is physiologically phosphorylated by the ecto-casein kinase 2 enzyme and the phosphorylation status is involved in the the shedding process regulation (Zimina et al., 2007). It has also been reported that circulating antibodies from BP patients'sera preferentially recognized the phosphoepitopes suggesting that posttranslational phosphorylation may be involved in the pathogenesis of BP (Zimina et al., 2008).
In line with these findings we show here that collagen XVII ectodomain shedding, which is a posttranslational event mediated by the enzymes from ADAM family, generates a novel epitope which is highly likely to be involved in the pathogenesis of autoimmune blistering skin diseases, such as BP or LAD.

EBA is a prototypical organ-specific autoimmune disease affecting the skin and the mucous membranes associated with autoantibodies against collagen VII (Mihai and Sitaru, 2007). The blister-inducing potential of autoantibodies against collagen VII has been shown in different ex vivo and animal models (Sitaru et al., 2007). Autoimmunity against collagen VII has been described in IBD, which may be clinically associated with EBA. Interestingly, collagen VII-specific autoantibodies are present in IBD patients, which do not show blistering skin disease (Hundorfean et al., 2010). In the present study, we also aimed to analyze collagen VII-specific autoantibodies, in larger groups of healthy blood donors or patients with EBA and other autoimmune diseases.

Serum immunoreactivity to the dermal side of the dermal–epidermal junction by salt-split skin and clinical and histological features of EBA are the most used diagnosis criteria. However, by indirect immunofluorescence one cannot exclude the reactivity with other basal membrane antigens on the dermal side such as P200 protein. Immunoblot using recombinant or native forms of collagen VII have been shown to be less sensitive than ELISA test (Chen et al., 1997a; Saleh et al., 2011). A possible explanation for the higher sensitivity of ELISA test can be that in immunoblot the protein is denaturated and doesn’t assess conformational epitopes.

To specifically detect collagen VII serum antibodies, immunoblot and ELISA tests have been developed (Chen et al., 1997a; Pendaries et al., 2010; Saleh et al., 2011). For the first collagen VII ELISA developed by Chen et al in 1997 a recombinant form of NC1 domain produced in mammalian cells has been used. This is a very sensitive test to detect antibodies directed to NC1 domain of collagen VII but doesn’t cover all potential antigenic sites residing in the NC2 domain as well as in the triple helical domain. Therefore, Pendaries et al., developed a new test using the full length form of collagen VII purified from conditioned medium of a recessive dystrophic epidermolysis bullosa keratinocyte cell line transduced with a COL7A1 retroviral vector. Although this seems to be the ideal ELISA for collagen VII antibodies detection, there are some drawbacks in using the full length form of the protein collagen VII mainly represented by the fact that a protein with a high molecular weight is difficult to produce and it is relatively instable. To overcome this problem, Saleh et al., established an ELISA using a mixture of two recombinant proteins: NC1 and
NC2 domains of collagen VII. NC1 protein was produced in mammalian system but they were not able to produce the NC2 protein in the same system which was therefore produced in bacteria.

To further improve the detection of collagen VII-specific autoantibodies, in the present study we have generated a chimeric antigen substrate containing virtually all autoepitopes, which have been reported in previous epitope mapping studies in patients.

Wet lab epitope mapping studies and our present in silico analysis have shown that the epitopes targeted by autoantibodies are localized within the NC1, NC2 and most probably the hinge region of the antigen. Therefore, to measure autoantibodies against collagen VII we have generated a chimeric protein containing all putative epitope-bearing regions of collagen VII, including its NC1, NC2 domains and the hinge fragment. The chimeric protein, which was produced in a human cell line to ensure optimal posttranslational modifications, is more stable compared with the full-length collagen and contains all the epitopes/regions in one copy per molecule. Hence, it ensures strict equimolar concentration of the NC1, NC2 and hinge regions respectively.

Possible isolated reactivity against hinge was reported so far only in 3 children with EBA (Tanaka, et al., 1997) and was apparently present in our relatively large cohort of adult patients only in one patient. This resulted in a slightly lower specificity of the ELISA using the fused NC1 and NC2 domains when compared with the form containing NC1, hinge and NC2 in the present study.

While the number of EBA patients with autoantibodies targeting only epitopes outside its NC1 and NC2 domains is not known, but certainly very low, the ELISA using recombinant collagen VII containing NC1, hinge and NC2 should significantly help detecting specific autoantibodies in these patients.

We found collagen VII-specific autoantibodies in 4% and 18% of patients with CD and UC, respectively. Our present results in CD patients are in line with our previous study showing collagen VII-specific autoantibodies by immunoblotting in 5.8% of CD and 5.8% of UC patients, in contrast to over 60% of CD patients initially reported (Chen et al., 2002b; Oostingh et al., 2005). Interestingly, we measured collagen VII-specific autoantibodies in a higher percentage of UC patients compared with 5.8% and 12.9%, that were reported in the previous studies (Oostingh et al., 2005; Chen et al., 2002c). The reason for this discrepancy is not known and future studies in larger number of IBD patients should help defining the prevalence of collagen VII-specific autoantibodies in these patients. While several hypotheses have been advanced, the induction of autoimmune response against collagen VII and the pathogenic significance of specific
autoantibodies in inflammatory bowel disease is still elusive.
Conclusions:

In the present study we were able to show that rabbits and sheep immunization with different fragments of murine type XVII collagen epitopes resulted in the production of polyclonal antibodies which stained the epidermal side of murine salt split skin and fixed the complement, two prerequisites for their *in vivo* pathogenicity.

Collagen XVII ectodomain shedding generates new epitopes on the resulting peptides which can be now recognized as non-self antigens by the adaptive immune system. Here we demonstrate that rabbit antibodies generated against a human collagen XVII neo-epitope induced dermal-epidermal separation of human skin when co-incubated with granulocytes demonstrating that collagen XVII ectodomain shedding can generate neo-epitopes which are pathogenic.

Despite its powerful scavenging effect of more than 85%, superoxide dismutase is not able to inhibit the blister-inducing capacity of autoantibodies. In contrast, specific inhibition of the myeloperoxidase could block the antibody-induced granulocyte-mediated dermal-epidermal separation.

Using a recombinant chimeric protein comprising all putative epitope-bearing regions of collagen VII, including its NC1, NC2 domains and the hinge fragment we developed a novel sensitive ELISA assay for detecting collagen specific antibodies in patients with EBA and inflammatory bowel disease. This immunoassay will be a useful tool for the sensitive and specific detection of anti-collagen autoantibodies in epidermolysis bullosa acquisita and other diseases associated with autoimmunity against collagen VII.
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