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der Universität Würzburg**

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**Untersuchungen zur Pathogenität von  
Autoantikörpern gegen Typ XVII Kollagen  
von Patienten mit Pemphigoid gestationis**

**Pathogenic relevance of autoantibodies to type  
XVII collagen from  
pemphigoid gestationis patients**

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## Abbreviations

Aa:	amino acid
BMZ:	basal membrane zone
BP:	bullous pemphigoid
BP180:	bullous pemphigoid antigen of 180-kDa / type XVII collagen
BP230:	bullous pemphigoid antigen of 230-kDa
BSA:	bovine serum albumin
DEJ:	dermal-epidermal junction
ELISA:	enzyme-linked immuno-assay
GST:	glutathione-S-transferase
HRP:	horseradish peroxidase
IF:	immunofluorescence
LAD-1:	linear IgA disease antigen-1
NC16A:	16th noncollagenous A domain
PBS:	phosphate-buffered saline
PG:	pemphigoid gestationis
SDS-PAGE:	sodium dodecyl sulfate-polyacrilamide gel electrophoresis
TBS:	Tris-buffered saline
TBST:	Tris-buffered saline plus Tween-20

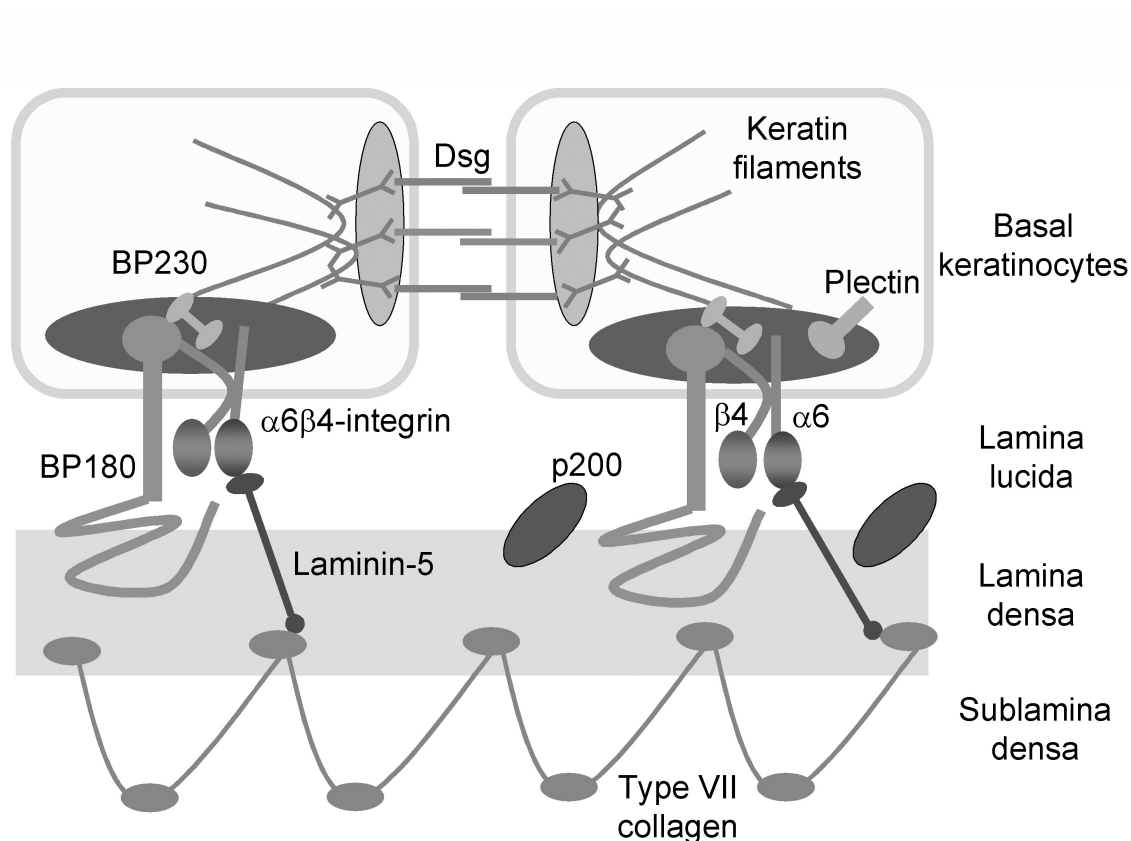


## Introduction

Organ-specific autoimmune diseases are characterized by T and B cells that initiate a reaction against defined self-proteins, which may result in tissue damage. In many of these conditions, the epitopes targeted by pathogenic T cells and autoantibodies have been extensively characterized in patients and experimental animals (Forsthuber et al. 2001; Kuwana et al. 2001; Sekiguchi et al. 2001; Tsunoda et al. 2003; Xie et al. 1997; Yang et al. 2002).

Autoimmune blistering skin diseases represent one group of organ-specific autoimmune disorders. They are clinically characterized by blisters and erosions and histologically classified as intraepidermal (pemphigus diseases) or subepidermal (pemphigoid diseases and epidermolysis bullosa acquisita), regarding the level of skin detachment. They are all associated with tissue-bound and circulating autoantibodies against structural elements of skin and mucosal surfaces.

Autoantigens recognized by autoantibodies in pemphigus patients are mostly components of the desmosomes, structures involved in the intercellular adherence among keratinocytes. These structures include desmogleins (Amagai et al. 1991; Karpati et al. 1993; Stanley et al. 1982; Wang et al. 1997), desmocollins (Hashimoto et al. 1997), plakoglobin (Korman et al. 1989), desmoplakins (Hashimoto et al. 1995; Oursler et al. 1992) and plectin (Aho et al. 1999; Proby et al. 1999). Non-structural proteins such as acetylcholine receptors have been also reported as autoantigens in this group of skin disorders (Nguyen et al. 2000).



**Figure 1. Autoantigens at the dermal-epidermal junction.** The different proteins recognized as autoantigens in subepidermal blistering diseases are depicted and located on the different layers of the basal membrane zone. BP230 and plectin are intracellular hemidesmosomal proteins. BP180 and  $\alpha6\beta4$ -integrin are transmembrane proteins of the hemidesmosomes, which project into the lamina lucida. BP230 interacts with both transmembrane proteins (Hopkinson and Jones 2000; Koster et al. 2003). BP180 intracellularly interacts with the  $\beta4$ -unit of  $\alpha6\beta4$ -integrin (Aho and Uitto 1998). Laminin 5, located in the lamina densa, acts as a bridge between  $\alpha6\beta4$ -integrin and type VII collagen (Falk-Marzillier et al. 1998; Rousselle et al. 1997), the major component of anchoring fibrils. Anchoring fibrils are responsible for the adherence of the basal membrane zone to the dermis (dermal collagens). The protein p200 is not yet fully characterized and localizes to the lower lamina lucida. In addition, desmosomes, which are structures responsible for the epidermal cell-cell adherence are also represented in the figure. In intraepidermal blistering diseases (pemphigus), patients present an autoimmune response against these desmosomal molecules, e.g. against desmogleins (Dsg).



In contrast, the autoimmune response in subepidermal blistering skin diseases is directed against multiple molecules of the dermal-epidermal junction (DEJ) (**Figure 1**): bullous pemphigoid antigen of 230-kDa (BP230) (Stanley et al. 1981), plectin (Laffitte et al. 2001), bullous pemphigoid antigen of 180-kDa (BP180) (Diaz et al. 1990; Giudice et al. 1992),  $\alpha 6\beta 4$ -integrin (Bhol et al. 2000; Bhol et al. 2001; Kumari et al. 2001; Tyagi et al. 1996), laminin 5 (Domloge-Hultsch et al. 1992; Kirtschig et al. 1995; Nousari et al. 1999), type IV collagen (Ghohestani et al. 2000), type VII collagen (so-called the epidermolysis bullosa acquisita antigen) (Woodley et al. 1984) and the not yet characterized p200 protein (Zillikens et al. 1996).

Pemphigoid gestationis (PG) is a subepidermal autoimmune blistering skin disease associated with pregnancy and early postpartum (Shimanovich et al. 2002a; Shornick et al. 1983). Disease usually starts during the second half of pregnancy or the first weeks of postpartum, with intense pruritus that precedes the onset of skin blisters (Shornick 1987). The clinical presentation is polymorphic (papules, plaques, targetoid lesions, vesicles and bullae) and blisters are not a constant finding (Ogilvie et al. 2000). Initial lesions are typically located on the abdomen and later generalize. Mucous membranes are rarely involved (Shimanovich et al. 2002b). The disease tends to spontaneously improve in the last stages of pregnancy, exacerbates after delivery and finally disappears without scarring (Shornick 1987). Flares may appear during subsequent pregnancies and hormonal contraceptive treatment (Holmes et al. 1983). Cases of PG in association with hydatiform mole (Tindall et al. 1981) and choriocarcinoma (do Valle Chiossi et al. 2000) have also been described.

An immunopathologic hallmark of PG is the linear deposition of C3 with or without IgG at the basement membrane zone (BMZ), as detected by direct immunofluorescence (IF) study of perilesional skin (Jordon et al. 1976; Katz et al. 1976). Circulating IgG autoantibodies can be identified in most PG patients using an indirect complement fixation IF technique (Jordon et al. 1976; Katz et al. 1976). By immunoelectron microscopy, deposition of immunoreactants is located just below the basal keratinocytes, on the upper layer of the lamina lucida (Shimanovich et al. 2002a). The autoimmune response in PG is mainly directed

against type XVII collagen, also called BP180 (Kelly et al. 1990; Morrison et al. 1988). Type XVII collagen is a component of hemidesmosomes (**Figure 1**), supramolecular structures that mediate adhesion of basal keratinocytes and certain other epithelial cells to the underlying basement membrane (Franzke et al. 2005). This glycoprotein consists of an N-terminal intracellular terminus (located in the hemidesmosomal plaque), a short transmembrane stretch and a long extracellular, rodlike region spanning the entire lamina lucida (Franzke et al. 2005). The different components consist of 466, 23 and 1008 amino acids (aa) in length, respectively (Franzke et al. 2005). The extracellular C-terminal region contains 15 collagenous (COL1-15) and 16 non-collagenous domains (NC1-NC16), some of which are N-glycosylated (Franzke et al. 2005). Confirmed ligands of type XVII collagen include: plectin, BP230 and  $\alpha 6\beta 4$ -integrin (Hopkinson et al. 1995; Hopkinson et al. 1998; Hopkinson and Jones 2000; Koster et al. 2003). Mutations in the human gene of type XVII collagen, COL17A1, may lead to rudimentary hemidesmosome formation and subsequent dermal-epidermal separation (McGrath et al. 1995). These structural alterations are responsible for the clinical hallmarks of junctional epidermolysis bullosa, a multi-phenotypic genetic disorder with mucocutaneous blistering disease, nail, hair and teeth abnormalities (Franzke et al. 2005).

In addition to IF studies, immunoblot and enzyme-linked immunosorbent assay (ELISA) are useful tools for the diagnosis of PG. Using recombinant forms of type XVII collagen/BP180, autoantibodies against this molecule were found in 93 and 86% of PG patients, as detected by immunoblot and ELISA, respectively (Sitaru et al. 2004). The ELISA system may be of special interest in order to guide treatment decisions in PG patients, since serum levels of autoantibodies to BP180 seem to parallel the clinical disease activity (Sitaru et al. 2004).

Autoimmunity to type XVII collagen associates not only with PG but also with other subepidermal autoimmune blistering skin diseases of the pemphigoid group, including bullous pemphigoid (BP) (Diaz et al. 1990; Labib et al. 1986), mucous membrane pemphigoid (Balding et al. 1996; Bernard et al. 1992; Bernard et al. 1990), lichen planus pemphigoides (Davis et al. 1991; Tamada et al. 1995; Zillikens et al. 1999a) and linear IgA disease (Zone et al. 1990; Zone et al. 1996;

Zone et al. 1998; Zillikens et al. 1999b). The pathogenic relevance of autoantibodies to type XVII collagen is supported by several lines of evidence: 1) the passive transfer of autoantibodies from mothers suffering from PG to the fetus induces transient skin blistering in the newborn (Chorzelski et al. 1976; Katz et al. 1977); 2) serum levels of autoantibodies to type XVII collagen correlate with disease activity in patients with BP and PG (Schmidt et al. 2000; Sitaru et al. 2004); 3) autoantibodies to type XVII collagen from patients with BP recruit leucocytes to the DEJ and induce dermal-epidermal separation in cryosections of human skin (Sitaru et al. 2002b); 4) rabbit antibodies generated against murine and hamster forms of type XVII collagen induce subepidermal blisters when passively transferred into neonatal mice or hamsters, respectively (Liu et al. 1993; Yamamoto et al. 2002). Studies using these *in vitro* and *in vivo* models suggested that the epitopes targeted by pathogenic autoantibodies map within the extracellular membrane-proximal domain of type XVII collagen, which is designated as the 16<sup>th</sup> non-collagenous domain (NC16A) in humans (Liu et al. 1993; Yamamoto et al. 2002; Sitaru et al. 2002b; Hofmann et al. 2002). In the murine experimental model of autoimmunity to type XVII collagen, a unique linear epitope of 9-12 aa is targeted by blister-inducing rabbit antibodies (Liu et al. 1995). However, the sequences of human and murine type XVII collagen are not identical (Liu et al. 1993). In addition, because of differences between the murine and human immune system (Mestas and Hughes 2004), the pathomechanisms of blister formation may not be identical in mice compared with patients. The pathogenic epitopes in humans have not been yet defined. The characterization of these epitopes may have profound implications on the treatment of PG and other subepidermal blistering diseases associated with autoantibodies to type XVII collagen. In particular, an immunoadsorption utilizing short peptides covering the pathogenic epitope(s) could be effective in controlling the course of disease.

## **Aim of the study**

In the present study, we aimed at defining the epitopes on type XVII collagen that are pathogenically relevant for blister formation in patients with PG, and therefore, provide the basis for the development of a peptide-specific immunoadsorption therapy. We used an *in vitro* model, in which preparations containing PG autoantibodies (serum, total IgG fraction, specific anti-NC16A affinity-purified immunoglobulins) are able to induce dermal-epidermal separation in cryosections of human skin when co-incubated with leucocytes from healthy donors. First, we performed an epitope-mapping study to define which epitopes of type XVII collagen are recognized by circulating PG autoantibodies. In a second set of experiments, we affinity-purified autoantibodies specific to these epitopes and analyzed their *in vitro* blister-inducing ability. Finally, we addressed the question whether preadsorption of PG sera against two synthetic peptides including both major antigenic determinants was able to abolish their split-inducing potential.

## Materials and methods

### *Patients*

Serum samples were obtained from patients with BP (n=10) and PG (n=23), before initiation of treatment, as well as from healthy donors (n=10). All pemphigoid patients were characterized by: (i) blistering skin disease; (ii) subepidermal cleavage by histopathology; (iii) linear IgG and/or C3 deposition along the DEJ by direct IF microscopy; (iv) circulating autoantibodies to the DEJ as revealed by indirect IF microscopy; (v) reactivity to type XVII collagen as detected by immunoblot analysis and ELISA (Sitaru et al. 2002a; Sitaru et al. 2002b). In addition, all PG patients were pregnant or in the early postpartum period. The reactivity pattern of those PG patients' sera that induced dermal-epidermal separation in the cryosections of human skin, was analyzed by immunoblotting using recombinant forms of BP180 and cell-derived BP180, BP230 and the soluble ectodomain of BP180 (LAD-1) (**Table I**). Antibody reactivity to BP230 was not detected in any of our PG patients' sera.

**Table I. Reactivity and fine molecular specificity of pemphigoid gestationis patients' autoantibodies**

Patients	BP180 NC16A (aa) <sup>a</sup>				LAD-1 <sup>b</sup>	ELISA <sup>c</sup>
	507-520	521-534	507-534	490-562		
PG1	+	+	+	+	-	111
PG2	+	-	+	+	-	117
PG3	+	-	+	+	-	63
PG4	+	-	+	+	-	112
PG5	+	-	+	+	-	53
PG6	+	-	+	+	-	1387
PG7	+	-	+	+	-	812
PG8	+	-	+	+	-	39
PG9	+	-	+	+	-	102
PG10	+	-	+	+	-	51
PG11	+	-	+	+	+	497
PG12	+	-	+	+	+	92
PG13	-	-	-	+	-	115
PG14	+	-	+	+	-	98
PG15	+	+	+	+	-	115
PG16	+	-	+	+	-	169
PG17	+	-	+	+	-	177
PG18	+	-	+	+	-	233
PG19	n/d	n/d	n/d	N/d	-	96

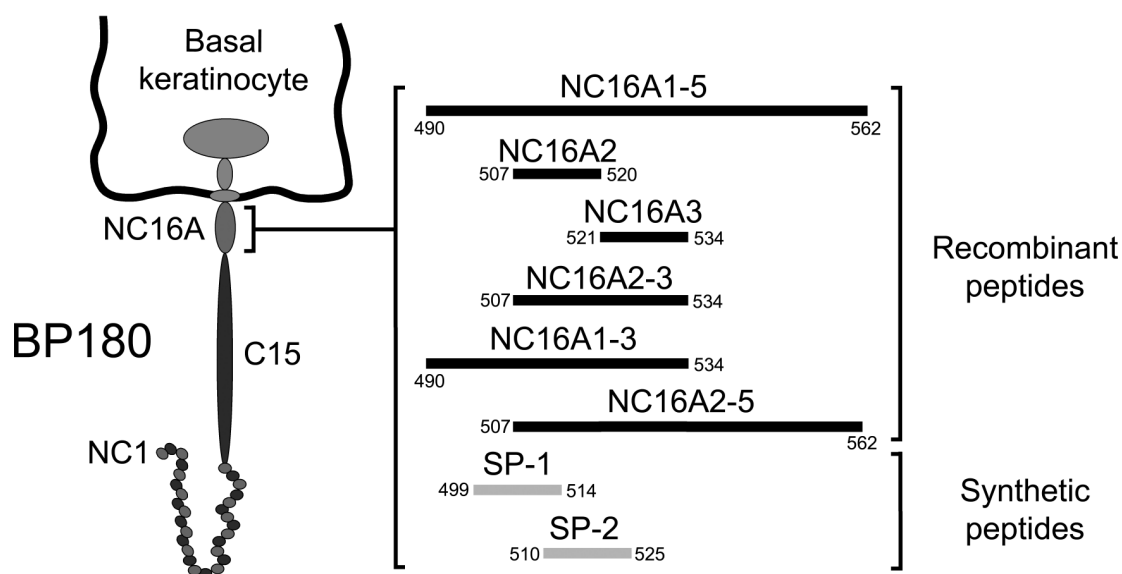
<sup>a</sup> Immunoblot reactivity with recombinant fragments covering different amino acid sequences of the NC16A domain of type XVII collagen/BP180.

<sup>b</sup> Immunoblot reactivity with the soluble ectodomain of type XVII collagen (LAD-1) from concentrated conditioned medium of cultured keratinocytes.

<sup>c</sup> ELISA using recombinant BP180 NC16A.

### **Preparation of recombinant and cell-derived forms of type XVII collagen**

Glutathione-S-transferase (GST) fusion proteins containing different fragments of the NC16A domain of type XVII collagen (**Figure 2**), including GST-BP180<sub>507-520</sub> (NC16A2), GST-BP180<sub>521-534</sub> (NC16A3), GST-BP180<sub>490-534</sub> (NC16A1-3), GST-BP180<sub>507-534</sub> (NC16A2-3), GST-BP180<sub>507-562</sub> (NC16A2-5), and GST-BP180<sub>490-562</sub> (NC16A1-5) were expressed in *Escherichia coli* DH5 $\alpha$  (Zillikens et al. 1997) and purified by glutathione-agarose immunoaffinity chromatography (Sitaru et al. 2005). The soluble shed ectodomain of type XVII collagen (LAD-1) was prepared from concentrated conditioned medium of cultured keratinocytes as described elsewhere (Zillikens et al. 1999b). The amino-terminus of LAD-1 corresponds to Leu<sup>524</sup> of the type XVII collagen sequence (Hirako et al. 2003). Cell-derived BP180 and BP230 were obtained from extracts of human cultured keratinocytes (Sitaru et al. 2002b).



**Figure 2. Recombinant and synthetic peptides of type XVII collagen (BP180) used in this study.** The ectodomain of type XVII collagen consists of a series of alternating collagenous (C, dark grey) and non-collagenous (NC, light grey) domains. The recombinant and synthetic forms of BP180 used in this study are depicted to the right. Recombinant peptides (NC16A1-5, NC16A2, NC16A3, NC16A2-3, NC16A1-3, NC16A2-5), represented by black bars, cover the whole

NC16A domain. Two 16-mer peptides (SP-1, SP-2), which are shown as grey bars, were synthesized to include the 2 major antigenic determinants and subsequently used for preadsorption experiments. Amino acid residue numbers are shown next to the bars. NC1, 1<sup>st</sup> non-collagenous domain; C15, 15<sup>th</sup> collagenous domain, NC16A, 16<sup>th</sup> non-collagenous A domain.

### ***Production of synthetic peptides***

Peptides bound to cellulose were automatically synthesized with an ASP 222-Spot Roboter (Intavis AG, Cologne, Germany) using the SPOT-synthesis technique (Frank 2002). For an initial characterization of epitopes, we synthesized 16-mer peptides with an offset of 2 aa covering a stretch of type XVII collagen delimited by aa 475 to 677. For a subsequent fine analysis of the epitopes, 16-mer peptides were synthesized with an offset of 1 aa residue covering a shorter sequence of type XVII collagen spanning aa 491 to 540. Two peptides SP-1 (aa 499-514) and SP-2 (aa 510-525) were synthesized and used for ELISA and preadsorption experiments (**Figure 2**).

### ***Immunofluorescence (IF) microscopy, immunoblotting and ELISA***

IF microscopy analyses followed standard protocols (Sitaru et al. 2002b). The complement-fixation test was performed as described (Sitaru et al. 2004; Sitaru et al. 2003). The recombinant proteins were subjected to 15% SDS-PAGE and electrophoretically transferred to nitrocellulose (Zillikens et al. 1997). Immunoblotting using recombinant and cell-derived proteins followed published protocols (Sitaru et al. 2002b; Zillikens et al. 1997). For epitope-mapping studies, equimolar amounts of the recombinant fragments were separated by SDS-PAGE and analyzed by immunoblotting using patients' sera (Chimanovitch et al. 1999). For a fine analysis of target epitopes, peptide spot-membranes were incubated with patients' sera (diluted 1:250 in TBS, pH 7.2) for 1 hour at room temperature and washed with TBST. Bound antibodies were visualized with a HRP-labeled anti-human IgG antibody (DAKOCytomation, Glostrup, Denmark) using diaminobenzidine as chromogenic substrate. ELISA using recombinant BP180



NC16A was performed following the manufacturer instructions (MBL Co, Nagoya, Japan). For ELISAs using synthetic fragments of type XVII collagen, 96-well plates (Nunc, Wiesbaden, Germany) were coated with the SP-1 and SP-2 peptides at a concentration of 20 µg/ml and treated with 1% BSA in TBS for 1 hour at room temperature to block unspecific binding. Patients' sera diluted 1:100 in TBS were incubated for 1 hour at room temperature with the peptide-coated wells, and bound antibodies were detected using a HRP-labeled anti-human IgG antibody and 3,3',5,5-tetramethylbenzidine as chromogenic substrate. Each serum was assayed in triplicate, at least in two independent experiments.

#### ***Complement source for immunofluorescence studies and complement inactivation of sera***

1 ml aliquotes of fresh frozen human serum from healthy volunteers were used as a source of complement. Complement inactivation of sera was achieved by heating at 56°C for 30 minutes (Gammon et al. 1982).

#### ***Affinity purification of total IgG and specific anti-BP180 autoantibodies***

Total IgG fraction was purified from serum using protein G chromatography as described (Sitaru et al. 2002a). Recombinant proteins GST-BP180<sub>507-520</sub>, GST-BP180<sub>490-534</sub>, and GST-BP180<sub>507-562</sub> were covalently coupled to 4% beaded agarose at pH 10 as described (Sitaru et al. 2002a; Sitaru et al. 2002b). Synthetic peptides were covalently coupled to CNBr-activated agarose (Sepharose 4B; Amersham Biosciences, Uppsala, Sweden) following the manufacturer instructions. Between 1.2 and 1.5 ml of each serum sample were incubated with the affinity matrices for 15-30 minutes at room temperature. Unbound antibodies were recovered by collecting the flow-through fraction and the first wash with 1-3 ml of PBS (pH 7.2). Antibodies bound to the affinity matrix were eluted with 0.1 M glycine buffer (pH 2.8) and the low pH was buffered with 1.5 M Tris-HCl. To achieve a complete preadsorption, this procedure was repeated 3-4 times. Both flow-through and eluted fractions were concentrated by ultrafiltration with Ultrafree 15 filters

(Millipore, Bradford, MA), under extensive washing with PBS (pH 7.2), to 1 ml and 0.2 ml, respectively. Reactivity of serum, flow-through, and eluted fractions was analysed by IF microscopy, immunoblotting and ELISA.

### ***Isolation of peripheral blood leucocytes***

Peripheral blood leucocytes from healthy volunteers were isolated by sedimentation gradient with dextran 500 followed by hypotonic lysis of red blood cells in 0.2% NaCl. Cells were harvested, washed twice in RPMI 1640 (Life Technologies, Karlsruhe, Germany) and resuspended in the same medium at a density of  $3 \times 10^7$  cells per ml. Cell suspension was kept on ice and cell viability was tested by trypan blue exclusion. Only preparations with a viability greater than 95% were used.

### ***Treatment of cryostat sections of normal human skin***

Neonatal human foreskin, obtained from routine circumcision, was washed in cold PBS, cut in small pieces and embedded in optimum cutting temperature compound (Sakura Finetek Europe B.B., Zoeterwoude, The Netherlands), and stored at  $-80^{\circ}\text{C}$ . Two to four cryosections of 6- $\mu\text{m}$  thickness were obtained and placed in the centre of glass microscope slides (Superfrost Plus microscope slides, Menzel-Gläser, Braunschweig, Germany). Autoantibody preparations (serum, total IgG fractions, elution and flow-through fractions from affinity purification) were diluted two-fold in PBS and incubated with the cryosections for 90-120 minutes at  $37^{\circ}\text{C}$  in a humidified air incubator containing 5%  $\text{CO}_2$ . After washing the sections twice in PBS, chambers with the skin sections were prepared as described (Sitaru et al. 2002b) and the leucocyte suspension injected into them. Preparations were incubated for 3 hours at  $37^{\circ}\text{C}$  in the same incubator and finally disassembled, washed 3 times in PBS for 10 minutes each, fixed in 10% formalin and stained with hematoxylin and eosin.

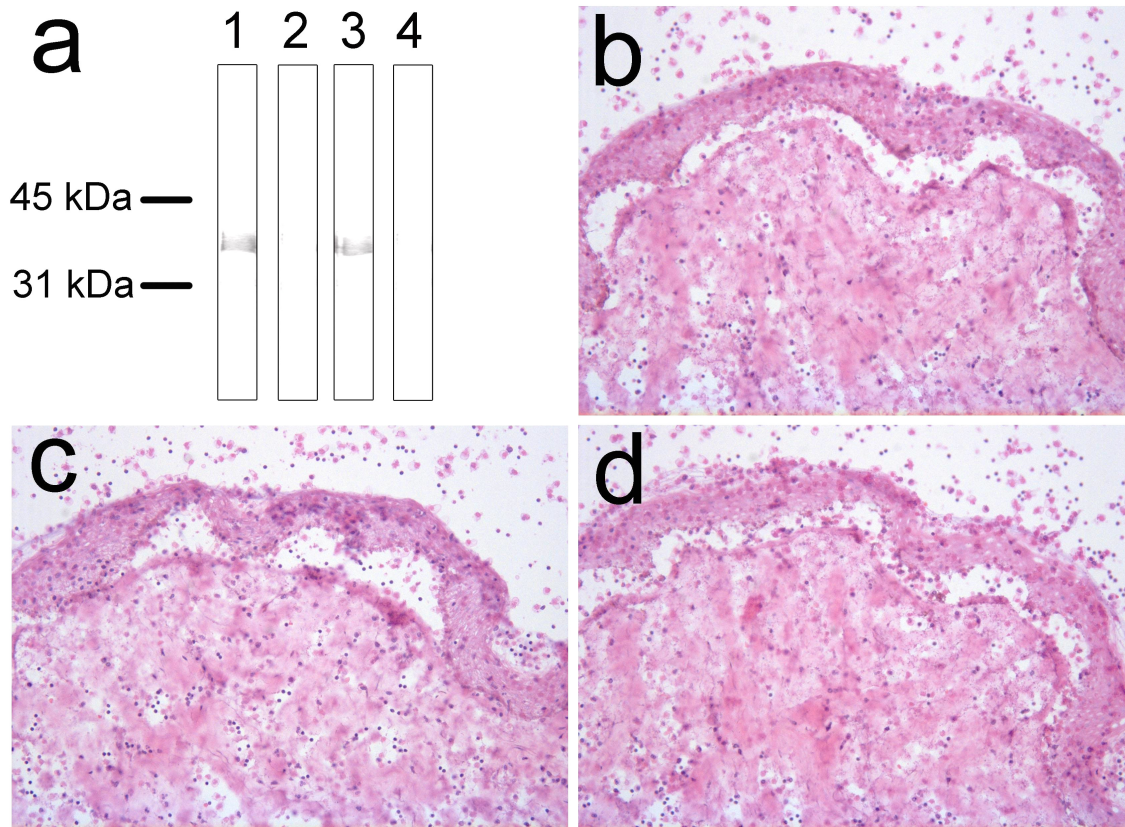
***Assessment of the blister-inducing potential of autoantibodies***

Blister-inducing capacity of patients' autoantibodies was evaluated using an *in vitro* model of autoantibody-induced dermal-epidermal separation (Shimanovich et al. 2004; Sitaru et al. 2002b). The extent of dermal-epidermal separation was scored as follows: + for 0-25%, ++ for 25-50%, +++ for 50-75%, and ++++ for 75-100% separation of the DEJ. Each experiment was repeated twice to demonstrate the reproducibility of the results.

## Results

### ***PreadSORption against the NC16A domain of type XVII collagen does not block autoantibody-induced dermal-epidermal separation in the majority of patients with bullous pemphigoid***

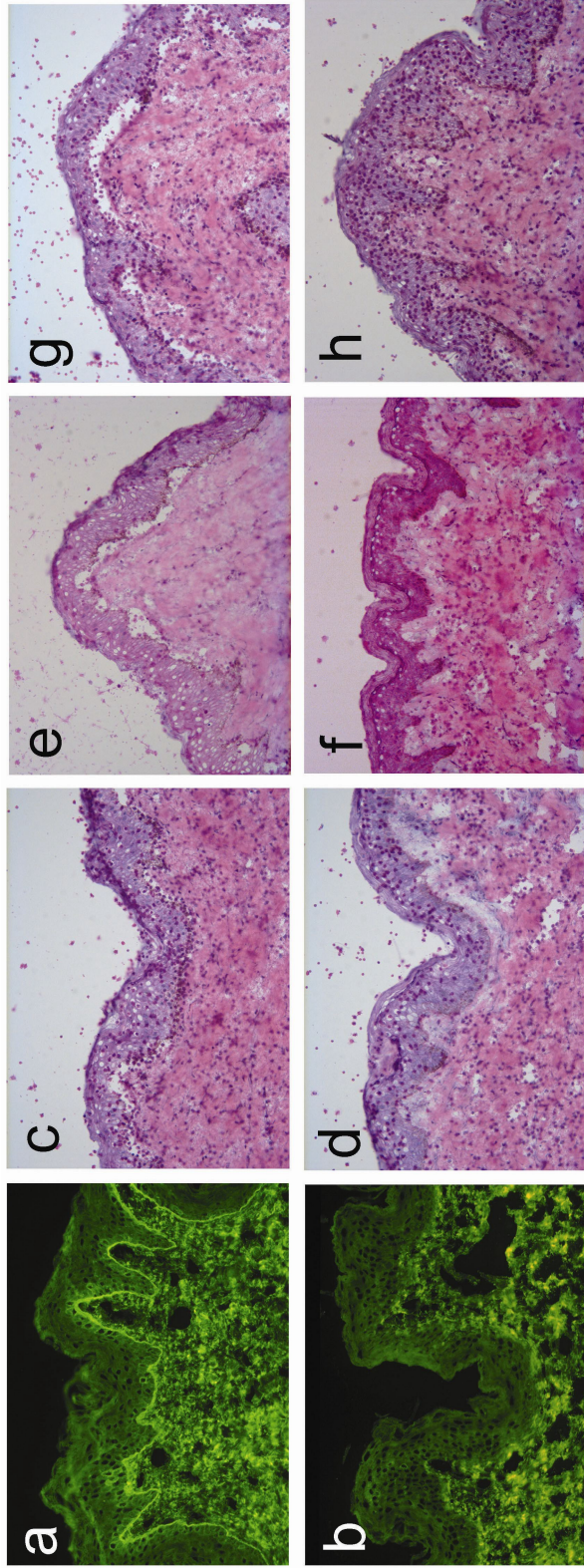
Antibodies from BP patients' sera (n=10) were purified against a recombinant form of the immunodominant NC16A domain of type XVII collagen. Total IgG from the flow-through fractions was further purified by protein G column chromatography. Specificity of antibodies eluted from NC16A coupled to agarose and of IgG purified from the flow-through fractions were analysed by IF microscopy and by immunoblotting using recombinant NC16A (**Figure 3a**). Like the original serum (**Figure 3b**), eluted autoantibodies specific to NC16A from all 10 patients induced subepidermal splits in cryosections of human skin (**Figure 3d**). Interestingly, in 7 of 10 patients, IgG preparations depleted of reactivity to the NC16A domain of type XVII collagen (flow-through fractions) continued to induce dermal-epidermal separation (**Figure 3c**). In the remaining 3 patients, flow-through fractions depleted of reactivity to NC16A lost their blister-inducing ability. These results indicate that, in the majority of BP patients, not only epitopes within but also outside the immunodominant region of type XVII collagen or in other BMZ components are targeted by blister-inducing autoantibodies. In contrast to BP, the antigenic determinants recognized by autoantibodies in PG are virtually restricted to the NC16A domain of type XVII collagen (Lin et al. 1999; Sitaru et al. 2003). Peptides covering this immunodominant region are available and suitable for immunoabsorption. Therefore, we used PG autoantibodies in order to map the pathogenically relevant epitopes in type XVII collagen.



**Figure 3. PreadSORption against the immunodominant region of type XVII collagen does not abolish the blister-inducing potential of autoantibodies in a majority of bullous pemphigoid patients.** Serum from a bullous pemphigoid patient was incubated with a protein cocktail consisting of GST-BP180<sub>490-534</sub> and GST-BP180<sub>507-562</sub> encompassing the entire NC16A domain covalently coupled to an agarose-beaded gel. The flow-through fraction was collected and bound antibodies eluted with 0.1 M glycine (pH 2,7). *a*, By immunoblotting using recombinant BP180 NC16A, IgG autoantibodies eluted from the column (*lane 3*) reacted, as the original serum (*lane 1*), with NC16A, whereas the flow-through fraction (*lane 2*) or normal serum (*lane 4*) showed no reactivity. Sections of human frozen skin were incubated with serum (*b*), flow-through (*c*) and eluted (*d*) fractions. All three fractions induced dermal-epidermal separation after addition of human normal leucocytes (hematoxylin and eosin, 200x). These results are representative for 7 of the 10 bullous pemphigoid sera included in this study.

***Pemphigoid gestationis autoantibodies induce leucocyte-dependent dermal-epidermal separation in cryosections of human skin***

Serum samples from PG patients (n=23) and IgG purified from 4 PG patients, as well as sera and IgG from patients with BP (n=4) and healthy volunteers (n=10), were incubated with cryosections of human skin in the presence of leucocytes isolated from the peripheral blood of healthy donors. Sera and IgG from 19 of 23 PG patients, as well as from all BP patients included, induced leucocyte recruitment to the DEJ and subepidermal splits in the skin cryosections (**Figure 4c,e**). Incubation of skin sections with serum and IgG from healthy controls did not result in leucocyte attachment to the epidermal basement membrane or dermal-epidermal separation (**Figure 4d,f**). To address the question whether the addition of complement increases the extent of dermal-epidermal separation in this model, we incubated cryosections, previously treated with PG sera, with 1) leucocytes and fresh serum, 2) leucocytes and complement-inactivated (heated) serum, or 3) with leucocytes 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 (data not shown). Therefore, further experiments did not include fresh serum as a source of complement.



**Figure 4. Autoantibodies to type XVII collagen in patients with pemphigoid gestationis induce dermal-epidermal separation in cryosections of human skin.** Serum IgG autoantibodies from a pemphigoid gestationis patient bound to the dermal-epidermal junction by immunofluorescence analysis (a), recruited leucocytes (c), and induced dermal-epidermal separation (e) in cryosections of human skin (hematoxylin and eosin, 200x). In contrast, IgG binding to the epidermal basement membrane by immunofluorescence microscopy (b), and leucocyte recruitment (d) or subepidermal splits (f) were not observed when the cryosections were incubated with a control serum (hematoxylin and eosin, 200x). Serum from a pemphigoid gestationis patient was incubated with a cocktail consisting of GST-BP180490-534 and GST-BP180507-562 encompassing the entire NC16A domain covalently coupled to an agarose-beaded gel. The flow-through fraction was collected and bound antibodies were eluted with 0.1 M glycine (pH 2,7). (g) Eluted antibodies specific for NC16A induced dermal-epidermal separation. (h) In contrast, patient's serum depleted of reactivity to NC16A lost its blister-inducing capacity.

***Preadsorption against the NC16A domain of type XVII collagen blocks the split formation induced by autoantibodies from pemphigoid gestationis patients***

Autoantibodies from PG patients (n=5) were purified against the entire recombinant NC16A domain of type XVII collagen. The reactivity and specificity of autoantibodies in the eluted and flow-through fractions were characterized by IF microscopy as well as ELISA and immunoblotting using recombinant proteins (**Table II**). When incubated with the cryosections, eluted autoantibodies specific to the NC16A domain of type XVII collagen from all PG patients induced subepidermal splits (**Figure 4g**), whereas serum fractions depleted of reactivity to this domain lost their blister-inducing ability (**Figure 4h**).



**Table II. Characterization of patients' serum fractions affinity-purified against recombinant fragments of type XVII collagen**

Patients	Fraction	BP180 <sub>490-562</sub>			BP180 <sub>507-520</sub>		
		IF <sup>a</sup>	ELISA <sup>b</sup>	DES <sup>c</sup>	IF <sup>a</sup>	ELISA <sup>b</sup>	DES <sup>c</sup>
PG1	Serum	1/40	111	+	1/40	111	++++
	Eluted fraction	1/80	234	++	1/20	84	++
	Flow-through	Neg	7	Neg	1/40	33	++
PG2	Serum	1/80	117	++	1/80	117	++
	Eluted fraction	>1/320	427	+++	1/80	154	++
	Flow-through	Neg	1	Neg	Neg	29	+
PG3	Serum	1/20	63	+	1/20	63	+++
	Eluted fraction	1/80	446	++	1/10	35	+
	Flow-through	Neg	0	Neg	Neg	15	++
PG4	Serum	1/40	112	+++	1/40	112	+++
	Eluted fraction	n/d	279	++++	n/d	58	++++
	Flow-through	Neg	16	Neg	Neg	52	++
PG5	Serum	1/40	53	++++	1/40	53	++++
	Eluted fraction	1/80	211	++++	1/40	81	+++
	Flow-through	Neg	1	Neg	Neg	15	++

Neg, negative; n/d, not done.

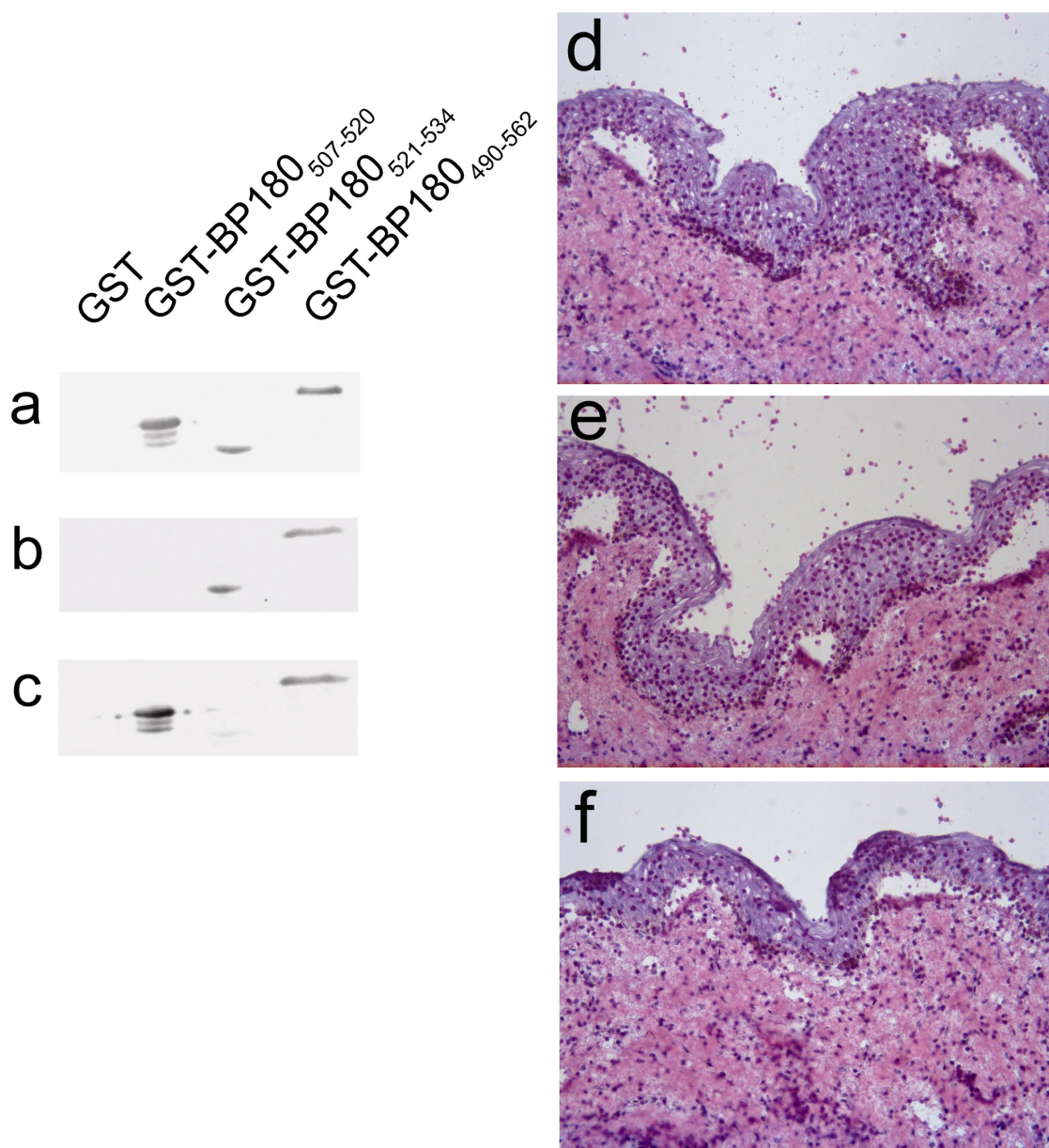
<sup>a</sup> Linear deposits of IgG on the epidermal side as demonstrated by immunofluorescence (IF) microscopy using sections of 1 M NaCl-split normal human skin.

<sup>b</sup> ELISA using recombinant BP180 NC16A.

<sup>c</sup> The fractions were incubated with cryosections of human skin in the presence of leucocytes. Dermal-epidermal separation (DES) was assessed as described in the Materials and Methods section.

***Binding of autoantibodies to more than one epitope of type XVII collagen results in subepidermal split formation***

To characterize more closely the epitopes targeted by pathogenic autoantibodies in PG, patients sera (n=5) were preadsorbed against a 14-amino acid stretch of type XVII collagen (aa 507-520; NC16A2) known to bear the main antigenic determinant(s) in PG (Chimanovitch et al. 1999; Lin et al. 1999). Reactivity and specificity of the eluted and flow-through fractions were analyzed by IF microscopy, ELISA using recombinant NC16A and immunoblotting using different recombinant fragments of NC16A, and the results are summarized in **Table II**. In general, preadsorption against NC16A2 led to a drastic reduction of reactivity to both the DEJ and to the NC16A domain of type XVII collagen as assessed by IF microscopy and ELISA, respectively. Interestingly, antibody preparations depleted of reactivity to the GST-BP180<sub>507-520</sub> (NC16A2) continued to induce subepidermal splits when incubated with the cryosections in all PG patients (**Table II, Figure 5e**). Antibodies specific to GST-BP180<sub>507-520</sub> from all patients caused dermal-epidermal separation (**Table II, Figure 5f**). These results indicate that blister-inducing autoantibodies in PG bind to more than one epitope on type XVII collagen.

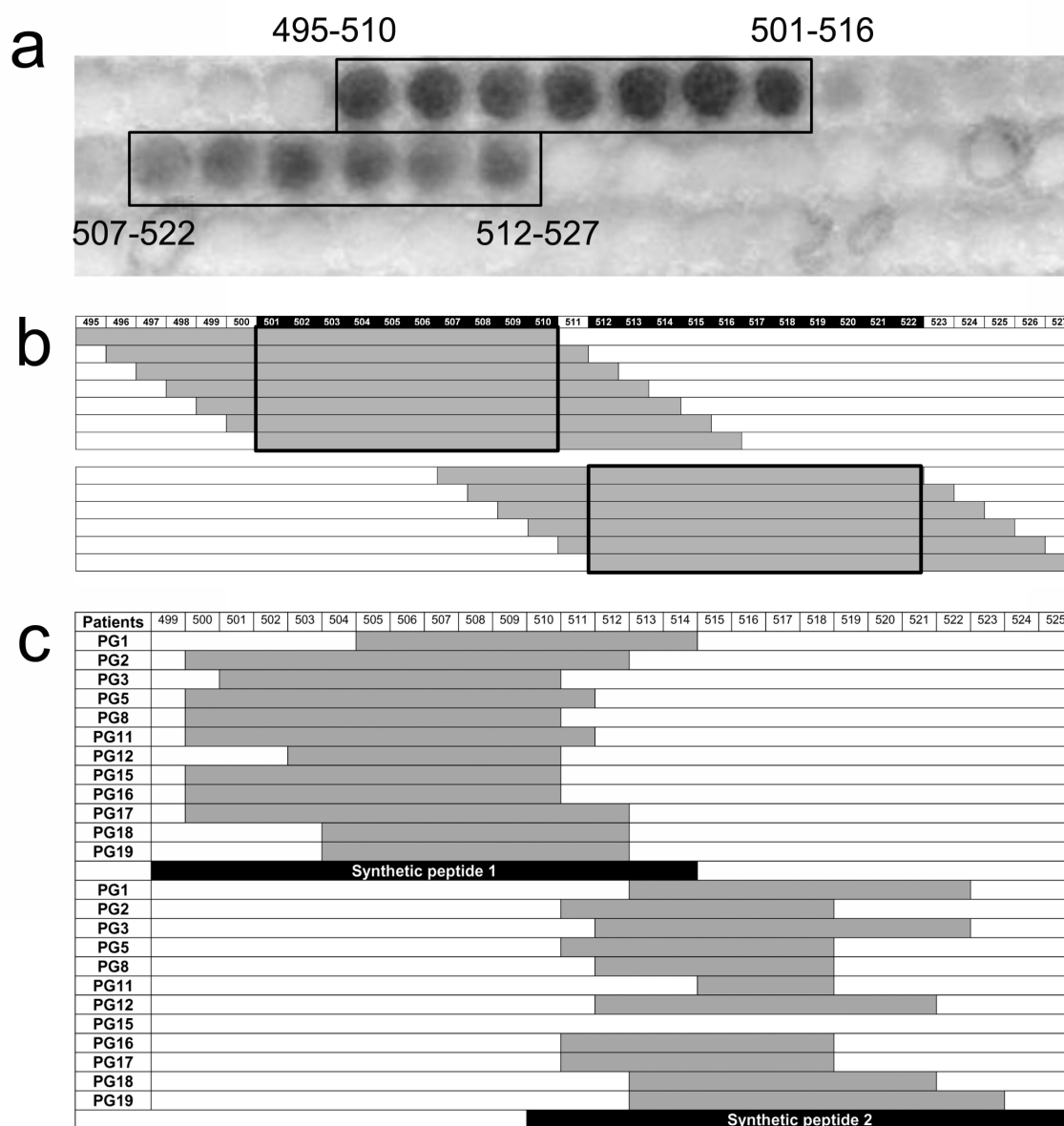


**Figure 5. Blister-inducing autoantibodies in pemphigoid gestationis target multiple epitopes of type XVII collagen.** Serum from a pemphigoid gestationis patient (PG1) was incubated with a recombinant protein covering aa 507-520 (NC16A2) of the type XVII collagen/BP180 sequence covalently coupled to an agarose-beaded gel. The flow-through fraction was collected and bound antibodies were eluted with 0.1 M glycine (pH 2,7). *a*, By immunoblot analysis using recombinant fragments of type XVII collagen, serum autoantibodies reacted with different epitopes localized within aa 490-562 of type XVII collagen. *b*, The flow-through fraction (preadsorbed serum) showed no reactivity to BP180<sub>507-520</sub>, but still reacted with BP180<sub>521-534</sub>. *c*, Conversely, eluted antibodies showed specificity to BP180<sub>507-520</sub> and did not react with BP180<sub>521-534</sub>. When incubated with the

cryosections in the presence of leucocytes, as the original serum did (d), both the flow-through fraction (e) depleted of reactivity to BP180<sub>507-520</sub> and eluted antibodies (f) specific for this BP180 fragment induced dermal-epidermal separation.

***Pemphigoid gestationis autoantibodies recognize two major epitopes within the NC16A domain of type XVII collagen***

We used synthetic peptides in order to map more precisely the epitopes recognized by PG autoantibodies. Overlapping peptides covering the NC16A domain were synthesized with an offset of 1 aa. Reactivity of PG sera (n=12) with these synthetic fragments was determined by dot-blot analysis. The results of these experiments are exemplified and summarized in **Figure 6**. PG patients' autoantibodies bound to 2 distinct epitopes on type XVII collagen (**Figure 6a,b**). The binding sites of PG autoantibodies are delimited by aa residues 500 – 514 and 511 – 523 of type XVII collagen (**Figure 6c**).



**Figure 6. Autoantibodies from pemphigoid gestationis patients bind to 2 distinct epitopes on type XVII collagen.** An array of consecutive 16-aa fragments covering aa 491-540 of type XVII collagen sequence were synthesized with an offset of 1 aa on cellulose membranes. (A) The membrane was incubated with patient's serum (PG3) and subsequently with a HRP-labeled anti-human IgG antibody. The reaction was developed using tetramethylbenzidine. PG autoantibodies bound to synthetic peptides (boxes) covering aa residues 495-516 and 507-527. (B) Schematic representation of the peptides recognized by patients' autoantibodies. The common sequence of these peptides is shown in boxes. (C) Summary of the epitopes recognized by epitope-mapping analyses of PG sera using overlapping synthetic peptides. The sequences of type XVII collagen

corresponding to the synthetic peptides used for preadsorption experiments are represented as black bars.

***PreadSORption against two synthetic peptides including the two major epitopes abolishes dermal-epidermal separation induced by pemphigoid gestationis autoantibodies***

Serum samples from PG patients (n=9) were preadsorbed against synthetic peptides containing the newly defined antigenic epitopes of type XVII collagen. The preadsorption was complete as revealed by ELISA using the synthetic peptides (data not shown). Reactivity and specificity of the eluted and flow-through fractions were analyzed by IF microscopy and ELISA using recombinant NC16A and are summarized in **Table III**. In 8 of 9 patients, in contrast to the original sera and eluted fractions, flow-through fractions contained no autoantibodies to the BMZ, including no complement-fixing autoantibodies, as assessed by IF microscopy (**Table III**). The levels of autoantibodies detected by ELISA using recombinant NC16A generally reflected the results by IF microscopy (**Table III**). Both original sera and eluted antibodies induced dermal-epidermal separation when incubated with cryosections of human skin (**Table III; Figure 7a,c**), whereas preadsorbed fractions of all but one PG serum lost their split-inducing ability (**Table III; Figure 7b**).

**Table III. Characterization of patients' serum fractions immunoaffinity-purified against two synthetic peptides of type XVII collagen<sup>a</sup>**

<b>Patients</b>	<b>Fraction</b>	<b>IF<sup>b</sup></b>	<b>ELISA<sup>c</sup></b>	<b>DES<sup>d</sup></b>
1	Serum	+	116	+++
	Eluted fraction	+	257	++++
	Flow-through	+	77	++
2	Serum	+	79	+
	Eluted fraction	+	69	+
	Flow-through	Neg	4	Neg
5	Serum	+	49	+++
	Eluted fraction	+	54	++
	Flow-through	Neg	14	Neg
8	Serum	+	46	++
	Eluted fraction	+	86	++
	Flow-through	Neg	5	Neg
11	Serum	+	105	+++
	Eluted fraction	+	269	++
	Flow-through	Neg	30	Neg
14	Serum	+/-	61	+/-
	Eluted fraction	+/-	36	+
	Flow-through	Neg	12	Neg
15	Serum	+	71	+
	Eluted fraction	+	143	++
	Flow-through	Neg	11	Neg
16	Serum	+	99	+
	Eluted fraction	+	123	++
	Flow-through	Neg	38	Neg
17	Serum	+	114	+
	Eluted fraction	+/-	134	++
	Flow-through	Neg	1	Neg

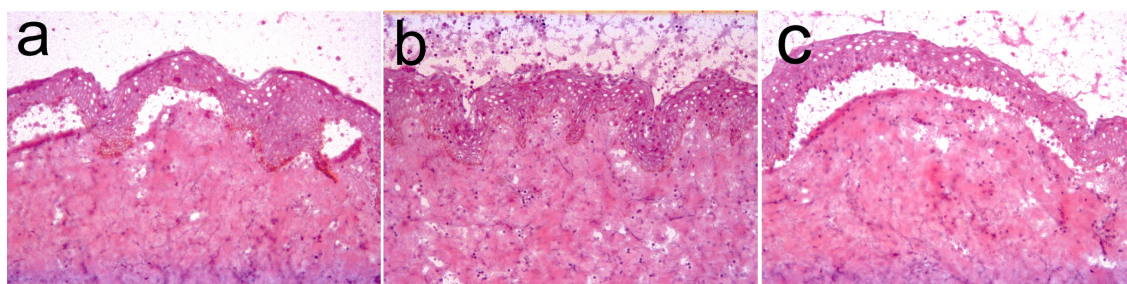
Neg, negative.

<sup>a</sup> Patients' sera were incubated with synthetic peptides covering aa 499-514 and aa 510-525 of type XVII collagen, covalently coupled to an agarose-beaded gel. The flow-through fraction was collected and bound antibodies eluted with 0.1 M glycine (pH 2,7).

<sup>b</sup> Evidence of C3 linear deposits on the epidermal side of 1 M NaCl-split normal human skin as demonstrated by immunofluorescence (IF) microscopy, when incubating antibody preparations (serum, eluted and flow-through fractions) and a complement source, as described in the Materials and Methods section.

<sup>c</sup> ELISA using recombinant BP180 NC16A.

<sup>d</sup> The fractions were incubated with cryosections of human normal skin in the presence of leucocytes. Dermal-epidermal separation (DES) was assessed as described in the Materials and Methods section.



**Figure 7. Preadsorption against synthetic peptides covering dominant B cell epitopes in pemphigoid gestationis abolishes blister-inducing ability.** Serum from a pemphigoid gestationis patient was incubated with two synthetic peptides covering both epitopes (aa 500-514 and 511-523) of the type XVII collagen sequence, previously immobilized in an agarose-beaded gel. The flow-through fraction was collected and bound antibodies were eluted with 0.1 M glycine (pH 2,7). In contrast to the original serum (a), the flow-through fraction (b), depleted of reactivity to the epitopes mentioned above, lost its blister-inducing ability. (c) Eluted antibodies induced subepidermal separation in cryosections of normal human skin.



## Discussion

Previous studies suggested that epitopes targeted by blister-inducing autoantibodies in pemphigoid diseases localize within the NC16A domain of type XVII collagen (Hofmann et al. 2002; Liu et al. 1993; Sitaru et al. 2002b; Yamamoto et al. 2002). In the murine experimental model of autoimmunity to type XVII collagen, blister-inducing antibodies bound to a single epitope within the extracellular membrane-proximal non-collagenous domain of type XVII collagen (Liu et al. 1993). In the present study, we show that pathogenic patients' autoantibodies target multiple epitopes on type XVII collagen. Specifically, 2 pathogenically relevant epitopes are located within the NC16A domain of type XVII collagen in PG patients, while blister-inducing autoantibodies in a majority of BP patients also target epitopes outside this immunodominant domain.

In a first set of experiments, we aimed at mapping the epitopes targeted by blister-inducing autoantibodies in BP patients. Based on previous epitope-mapping data (Giudice et al. 1993; Matsumura et al. 1996; Zillikens et al. 1997) and on the initial functional data using the cryosection model (Sitaru et al. 2002b), we hypothesized that pathogenically relevant epitopes localize within the NC16A immunodominant region of type XVII collagen. Interestingly, preadsorption of BP sera against a recombinant form of the entire NC16A region abolished the blister-inducing capacity in only about 1/3 of the patients. These data indicate that autoantibodies also targeting epitopes outside the immunodominant NC16A domain induce dermal-epidermal separation. Overlapping recombinant fragments covering the full-length of type XVII collagen, which are water-soluble and could be purified in sufficient amounts to be used for preadsorption experiments, are currently not available. Therefore, a systematic functional characterization of the epitopes recognized by blister-inducing autoantibodies in BP was not feasible. In contrast to BP, the autoimmune response in PG patients seems to be largely restricted to the N-terminal portion of the extracellular domain of type XVII collagen (Lin et al. 1999; Sitaru et al. 2003). Different recombinant fragments of this portion of the NC16A

domain were available and could be used for preadsorption studies (Zillikens et al. 1997). These reasons brought us to use sera from PG patients to characterize the pathogenic epitopes on type XVII collagen. As it was already described in BP (Sitaru et al. 2002b), PG autoantibodies induced dermal-epidermal separation when incubated with skin cryosections in the presence of leucocytes. However, in contrast to BP autoantibodies, preadsorption against a recombinant form of the entire NC16A domain of type XVII collagen successfully abolished the blister-inducing capacity of autoantibodies in all PG patients.

Previous epitope-mapping studies using recombinant forms of type XVII collagen revealed that autoantibodies from a majority of PG patients exclusively bind to a sequence corresponding to aa residues 507-520 (NC16A2) (Chimanovitch et al. 1999; Lin et al. 1999). Bearing this in mind, further experiments addressed the question whether preadsorption against this stretch of type XVII collagen abolishes the blister-inducing potential of PG autoantibodies. Interestingly, however, in all PG patients, both eluted anti-NC16A2 autoantibodies and serum fractions depleted of reactivity to this peptide induced *in vitro* dermal-epidermal separation. These results strongly suggested that blister-inducing autoantibodies recognize multiple epitopes within the NC16A domain of type XVII collagen.

To further characterize the pathogenically relevant targets of PG autoantibodies, we generated overlapping synthetic peptides covering the N-terminal sequence of the extracellular domain of type XVII collagen. Dot-blot analysis using these peptides clearly demonstrated that PG autoantibodies react with 2 distinct epitopes. Indeed, preadsorption against these 2 peptides, each containing 1 of the 2 newly defined epitopes, drastically reduced the reactivity of PG sera as measured by IF microscopy, immunoblotting and ELISA. Most importantly, serum fractions devoid of reactivity to the newly characterized epitopes lost their split-inducing potential. The location of these newly defined epitopes explains why our first preadsorption attempt against a recombinant fragment corresponding to aa residues 507-520 of type XVII collagen (NC16A2) did not abolish the pathogenic potential of autoantibodies in PG patients.

In the murine model of autoimmunity to type XVII collagen, pathogenic rabbit antibodies were shown to bind a unique epitope of 9-12 aa in length located within the N-terminal juxtamembranal extracellular region of type XVII collagen (Liu et al. 1995). This region corresponds in humans to the immunodominant NC16A domain. Taken together, these previous data and our present results indicate that the NC16A region is a hot spot for pathogenically relevant epitopes in PG and in a subgroup of patients with BP.

Our experiments demonstrate that blister-inducing autoantibodies bind to 2 epitopes of type XVII collagen in PG. In addition, our results show that the autoclavable synthetic peptides generated for this study are suitable for preadsorbing pathogenic autoantibodies. Antigen-specific immunoadsorption using short synthetic peptides has been devised and/or applied in other autoantibody-mediated diseases, including myasthenia gravis and dilated cardiomyopathy (Psaridi-Linardaki et al. 2005; Ronspeck et al. 2003; Wallukat et al. 2002). In patients with dilated cardiomyopathy, immunoadsorption using peptides that mimic the autoantibody-binding epitopes of the  $\beta$ 1-adrenergic receptor significantly lowered the levels of pathogenic antibodies (Jahns et al. 2004) and improved cardiac function (Wallukat et al. 2002). Since clinical improvement parallels the reduction of the levels of autoantibodies to type XVII collagen in PG patients (Sitaru et al. 2004), an antigen-specific immunoadsorption using these synthetic peptides is feasible. With regard to BP, further studies defining the pathogenically relevant epitopes are needed; these epitopes could be then used to preadsorb the blister-inducing potential of BP autoantibodies and to finally develop an antigen-specific immunoadsorption.

## Conclusion

This study demonstrates that autoantibodies of PG patients inducing *in vitro* dermal-epidermal separation bind to two main epitopes on type XVII collagen. Preadsorption experiments with two immobilized synthetic peptides including both major determinants successfully abolished this pathogenic potential. Our findings provide the rationale to develop an antigen-specific peptide-based immunoabsorber for the treatment of PG.

## Summary

Pemphigoid gestationis (PG) and bullous pemphigoid (BP) are subepidermal autoimmune blistering diseases characterized by self-reactive T and B cells specific for the transmembrane hemidesmosomal protein type XVII collagen/BP180. Major T and B cell epitopes are located within the immunodominant 16<sup>th</sup> non-collagenous domain A (NC16A) of type XVII collagen. It has been suggested that pathogenically relevant autoantibodies also bind to this immunodominant region.

The aim of this study was to map the epitopes targeted by blister-inducing human autoantibodies. For this purpose, we used an *in vitro* model of autoantibody-induced leucocyte-dependent dermal-epidermal separation. In contrast to the majority of patients with BP (7 of 10), preadsorption against a recombinant form of the NC16A region abolished the blister-inducing potential of autoantibodies from all PG patients tested (n=5). Using overlapping synthetic peptides, we demonstrate that PG autoantibodies bind to 2 defined epitopes within the NC16A region (aa 500-514 and aa 511-523). Preadsorption using an affinity matrix containing these two epitopes completely abolished dermal-epidermal separation induced by PG autoantibodies (in 8 of 9 patients). These findings provide new insights into the pathogenesis of pemphigoid diseases and should prove helpful for the development of an antigen-specific immunoadsorption therapy in PG.

## Zusammenfassung

Pemphigoid gestationis (PG) und bullöses Pemphigoid (BP) sind subepidermal Blasen bildende Autoimmundermatosen, die durch autoreaktive T- und B-Zellen gegen das hemidesmosomale Transmembranprotein Kollagen Typ XVII / BP180 gekennzeichnet sind. Die Haupt T- und B-Zell Epitope liegen in der immundominanten, nicht-kollagenösen 16. Domäne A (NC16A) des Kollagen Typ XVII. Man ging davon aus, dass auch die pathogenen Autoantikörper gegen diese immundominante Region gerichtet sind. Das Ziel dieser Studie war es, die pathogenetisch relevanten Epitope beim PG zu kartieren. Hierfür wurde ein *in vitro*-Modell eingesetzt, in dem Autoantikörper Leukozyten-abhängig eine dermo-epidermale Spaltbildung induzieren. Im Gegensatz zu einem Großteil der Patienten mit BP (7 von 10) führte die Präadsorption gegen eine rekombinante Form der NC16A-Region zum Verlust des Blasen induzierenden Potentials aller untersuchten PG-Seren (n=5). Mithilfe überlappender synthetischer Peptide konnten wir zeigen, dass die Autoantikörper im Serum von PG Patienten an zwei definierte Epitope innerhalb der NC16A-Region (aa 500-514 und aa 511-523) binden. Die Präadsorption gegen eine Affinitätsmatrix, an die diese 2 Epitope gebunden waren, führte zum Verlust der dermo-epidermalen Spaltbildung durch PG-Autoantikörper (in 8 von 9 Patienten). Diese Ergebnisse geben weitere Einblicke in die Pathogenese der Pemphigoid-Erkrankungen und dürften zur Entwicklung Antigen-spezifischer Immunadsorptionstherapien des PG beitragen.

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