表 1:抗表皮基底膜部抗体を示す自己免疫性水疱症の分類と抗原

| 病名 | イムノグロブリン | 抗原蛋白 |
|---|---|---|
| 水疱性類天疱瘡 | IgG | BP230、BP180 |
| 妊娠性疱疹 | IgG | BP180 |
| 粘膜類天疱瘡（MMP） | | |
| 抗 BP180 型 MMP | IgG/IgA | BP180（C 末端部） |
| 抗ラミニン 332 型 MMP | IgG | ラミニン 332<br>（ラミニン 5、エピリグリン） |
| 抗 p165 型 MMP | IgG | 未知 p168 |
| 眼型 MMP | IgA/IgG | インテグリンベータ４、LAD-1 |
| 口腔粘膜型 MMP | IgG/IgA | インテグリンアルファ６ |
| ジューリング疱疹状皮膚炎 | IgA | 表皮トランスグルタミナーゼ |
| C3 疱疹状皮膚炎（仮称） | C3 | 未知（なし？） |
| 線状 IgA 水疱性皮膚症 | | |
| Lamina lucida 型 | IgA | 97kDa/120kDa LAD-1 |
| Sublamina densa 型 | IgA | 未知<br>(VII 型コラーゲン、<br>ラミニンガンマ１サブユニット) |
| 後天性表皮水疱症 | IgG | VII 型コラーゲン |
| 水疱性 SLE | IgG | VII 型コラーゲン |
| 抗ラミニン・１類天疱瘡 | IgG | ラミニンガンマ１サブユニット |

下線：抗原未同定

# Ⅲ．研究成果の刊行に関する一覧表

## 研究成果の刊行に関する一覧表

雑誌


1 Csorba K, Sesarman A, Oswald E, Feldrihan V, Fritsch A, Hashimoto T, Sitaru C. Cross-reactivity of autoantibodies from patients with epidermolysis bullosa acquisita with murine collagen VII. Cell Mol Life Sci. 67:133-1351, 2010.

2 Schmidt E, Dähnrich C, Rosemann A, Probst C, Komorowski L, Saschenbrecker S, Schlumberger W, Stöcker W, Hashimoto T, Bröcker EB, Recke A, Rose C, Zillikens D. Novel ELISA systems for antibodies to desmoglein 1 and 3: correlation of disease activity with serum autoantibody levels in individual pemphigus patients. Exp Dermatol 19(5): 458–463, 2010.

3 Hashimoto T, Dainichi T, Ohyama B, Hamada T, Ishii N, Sato N, Tanigawa O, Nakayama J, Amano S, Nishiyama T, Karashima T, Nakama T, Yasumoto S. A case of antilaminin 332 mucous membrane pemphigoid showing a blister on the bulbar conjunctiva and a unique epitope on the alpha3 subunit.Br J Dermatol. 2010 Feb 25. [Epub ahead of print]

4 Hashimoto T, Hamada T, Dainichi T, Ishii N, Karashima T, Nakama T, Yasumoto S. How Does Intramolecular Epitope Spreading Occur in BPAG2 (BP180)? J Invest Dermatol 130(4):924-926,2010.

5 Dainichi T, Ohyama B, Ishii N, Yamaguchi Z, Yasumoto S, Hashimoto T. Refractory oral ulcers with multiple immunoglobulin G/immunoglobulin A autoantibodies without skin lesions. J Am Acad Dermatol. 62(4):712-715,2010.

6 Ishii N. Hamada T, Dainichi T, Karashima T, Nakama T, Yasumoto S, Zillikens D, Hashimoto T. Epidermolysis bullosa acquisita: What's new? J Dermatol 37:220-230,2010

7 Dainichi T, Koga H, Tsuji T, Ishii N, Ohyama B, Ueda A, Natsuaki Y, Karashima T, Nakama T, Yasumoto S, Zillikens D, Hashimoto T: From anti-p200 pemphigoid to anti-laminin $\gamma$ 1 pemphigoid. J Dermatol 37:231-238,2010

8 Endo Y, Kato M, Kitoh A, Kore-Eda S, Fukuda S, Hashimoto T, Toda K, Miyachi Y, Utani A. Pemphigoid without Mucous Involvement Showing Autoantibodies against Laminin-332 Gamma 2 Subunit. Br J Dermatol. 2010 Jun 25. [Epub ahead of print]

9 Groth S, Recke A, Vafia K, Ludwig RJ, Hashimoto T, Zillikens D, Schmidt E. Development of a simple ELISA for the detection of autoantibodies in anti-p200 pemphigoid. Br J Dermatol. 2010 Sep 21. [Epub ahead of print]

10 Suda-Takayanagi T, Hara H, Ohyama B, Hashimoto T, Terui T. A case of pemphigoid vegetans with autoantibodies against both BP180 and BP230 antigens. J Am Acad Dermatol 64(1):206-8, 2011.

11 Koga H, Hamada T, Ishii N, Fukuda S, Sakaguchi S, Nakano H, Tamai K, Sawamura D, Hashimoto T. Exon 87 skipping of the COL7A1 gene in dominant dystrophic epidermolysis bullosa. J Dermatol. 2010 Sep 20. [Epub ahead of print]

12 Morita K, Fujisawa A, Yagi Y, Makiura M, Fukuda S, Ishii N, Ohyama B, Hashimoto T. Immunoglobulin A anti-BP230 autoantibodies in linear immunoglobulin A/immunoglobulin G bullous dermatosis. J Dermatol. 2011 Mar 2. [Epub ahead of print]

13 Minato H, Ishii N, Fukuda S, Wakasa T, Wakasa K, Sogame R, Hashimoto T, Horiguchi Y. Heterogeneity of Brunsting-Perry type pemphigoid: A case showing blister formation at the lamina lucida, immune deposition beneath the lamina densa and autoantibodies against the 290-kD polypeptide along the lamina densa. J Dermatol. 2011 Mar 2. [Epub ahead of print]

14 Tsuruta D, Hashimoto T, Hamill KJ, Jones JC. Hemidesmosomes and focal contact proteins: Functions and cross-talk in keratinocytes, bullous diseases and wound healing. J Dermatol Sci 62(1):1-7. 2011.

15 Ishii N, Recke A, Mihai S, Hirose M, Hashimoto T, Zillikens D, Ludwig RJ. Autoantibody-induced intestinal inflammation and weight loss in experimental epidermolysis bullosa acquisita. J Pathol. 2011 Jan 10. [Epub ahead of print]

16 Fukuda S, Tsuruta D, Uchiyama M, Mitsuhashi Y, Kobayashi H, Ishikawa T, Ohyama B, Ishii N, Hamada T, Dainichi T, Karashima T, Nakama T, Yasumoto S, Hashimoto T. Brunsting-Perry type pemphigoid with immunoglobulin G autoantibodies to laminin-332, BP230 and desmoplakins I/II. Br J Dermatol. 2011 Apr 1. [Epub ahead of print]

17 Saleh MA, Ishii K, Kim YJ, Murakami A, Ishii N, Hashimoto T, Schmidt E, Zillikens D, Shirakata Y, Hashimoto K, Kitajima Y, Amagai M. Development of NC1 and NC2 domains of Type VII collagen ELISA for the diagnosis and analysis of the time course of epidermolysis bullosa acquisita patients. J Dermatol Sci. 2011 Mar 16. [Epub ahead of print]

# Ⅳ. 研究成果の刊行物・別刷

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Cellular and Molecular Life Sciences

RESEARCH ARTICLE

# Cross-reactivity of autoantibodies from patients with epidermolysis bullosa acquisita with murine collagen VII

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**Abstract** The pathomechanism of antibody-mediated tissue damage in autoimmune diseases can be best studied in experimental models by passively transferring specific autoantibodies into animals. The reproduction of the disease in animals depends on several factors, including the cross-reactivity of patient autoantibodies with the animal tissue. Here, we show that autoantibodies from patients with epidermolysis bullosa acquisita (EBA), a subepidermal autoimmune blistering disease, recognize multiple epitopes on murine collagen VII. Indirect immunofluorescence microscopy revealed that EBA patients' IgG cross-reacts with mouse skin. Overlapping, recombinant fragments of murine collagen VII were used to characterize the reactivity of EBA sera and to map the epitopes on the murine antigen by ELISA and immunoblotting. The patients' autoantibody binding to murine collagen VII triggered pathogenic events as demonstrated by a complement fixing and an ex vivo granulocyte-dependent dermal–epidermal separation assay. These findings should greatly facilitate the development of improved disease models and novel therapeutic strategies.

**Keywords** Autoimmunity · Autoantigen · Basement membrane zone · Collagen · Extracellular matrix

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## Introduction

Autoantibodies cause tissue damage in a group of autoimmune diseases. The demonstration of autoantibody pathogenicity and the characterization of the underlying mechanisms require the reproduction of the disease by the passive transfer of specific antibodies into laboratory animals. Thus, the pathogenic potential of autoantibodies in several autoimmune diseases has been established by the passive transfer of the IgG serum fraction purified from patients into mice or rats [1–7].

Epidermolysis bullosa acquisita (EBA), a severe chronic subepidermal blistering disease of skin and mucous membranes, is characterized by tissue-bound and circulating IgG antibodies to the dermal–epidermal junction [8]. Patients' serum autoantibodies bind to the 290-kDa collagen VII, the major component of anchoring fibrils [8, 9]. Autoantibodies in the majority of EBA patients recognize different epitopes within the noncollagenous (NC) 1 and 2 domains of collagen VII [8–11]. The pathogenic relevance of antibodies against collagen VII is supported by compelling evidence: (1) EBA autoantibodies were shown to recruit and activate leucocytes ex vivo, resulting in dermal–epidermal separation in cryosections of human skin [12]; (2) antibodies against collagen VII induce subepidermal blisters when passively transferred into mice [5, 13]; and

(3) immunization with recombinant autologous collagen VII induces an autoimmune response to this protein, resulting in a blistering phenotype closely resembling human EBA [14].

The replication of the major clinical, histo- and immunopathological features of human EBA by the passive transfer of antibodies specific to collagen VII into mice greatly facilitated the dissection of the pathophysiology of blister formation in this disease [15–17]. The existing mouse models reproducing the blister formation in experimental EBA use the passive transfer of IgG antibodies from rabbits immunized against murine collagen VII or EBA patients' IgG into animals. While these studies suggest that antibodies directed against different epitopes of the NC1 domain of collagen VII are pathogenic, further pathogenic epitopes may exist, and their relative contribution for blistering in EBA has not yet been addressed. Using patients' autoantibodies for the passive transfer experiments in mice has several advantages over rabbit antibodies, especially the fact that patient autoantibodies already possess the "ideal" specificity and effector functions. Major disadvantages of using patients' autoantibodies in mice include the limited availability of patient autoantibodies as well as the fact that the degree of cross-reactivity may be low and the epitope recognition pattern may differ substantially for the murine compared with the human antigen [18].

Autoantibodies from a few EBA patients were shown to cross-react with murine skin [5, 13]. However, autoantibodies from other patients bound poorly or not at all to mouse skin [5]. In addition, the epitopes on the murine antigen recognized by EBA autoantibodies have not yet been characterized. This information is highly relevant for the development of standardized reagents such as blister-inducing human monoclonal antibodies, which could replace EBA patient autoantibodies. Therefore, the aim of the present study was to systematically analyze the degree of cross-reactivity of autoantibodies with murine collagen VII in a large number of patients. For this purpose, we analyzed the reactivity of patients' autoantibodies with murine skin by indirect immunofluorescence (IF) microscopy. Subsequently, recombinant forms of murine collagen VII covering the NC1 and NC2 domains of the protein were generated. The reactivity of EBA sera with murine collagen VII was characterized by ELISA using these recombinantly expressed protein fragments. Further, the recognition pattern of murine collagen VII epitopes by patients' autoantibodies was characterized by immunoblotting and ELISA. Finally, the pathogenic potential of patients' autoantibodies binding to murine skin has been assessed by a complement-fixation test and by the ex vivo autoantibody-induced dermal–epidermal separation assay.

## Materials and methods

### Human sera

Serum samples were obtained from patients with EBA ($n = 35$) prior to the initiation of treatment. Patients were characterized by: (1) blisters on the skin; (2) linear deposits of IgG at the DEJ, as shown by direct IF microscopy; (3) circulating IgG autoantibodies binding to the dermal side of 1 mol/l of NaCl-split human skin, as shown by indirect IF microscopy; and (4) immunoblot reactivity with dermal and/or recombinant collagen VII. The distribution of IgG subclasses in serum of EBA patients ($n = 19$) was examined by indirect immunofluorescnce microscopy, using human salt-split skin sections. The results of this analysis are summarized in the electronic supplementary material, ESM, Table 2. EBA patients' autoantibodies binding to the dermal side of the salt-split skin belonged to all four IgG subclasses. As expected, the predominant subclass was IgG4, followed by IgG1 and IgG2, while IgG3 autoantibodies were detected in few EBA patients. For the experiments conducted, we obtained approval from the Ethics Committee of the Medical Faculty of the University of Freiburg, Freiburg, Germany (Institutional Board Projects no. 318/07 and 407/08). We obtained informed consent from patients whose material was used in the study, in adherence to the Helsinki principles.

### Cell culture

PAM 212 murine keratinocytes [19] were cultured in DMEM (CCPro) supplemented with 10% fetal calf serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Biochrom).

### Heterologous expression of recombinant forms of collagen VII in bacteria

The cDNA sequences coding for six overlapping fragments of the NC1 and one fragment corresponding to the NC2 domain of collagen VII were cloned into prokaryotic expression vectors and expressed in *Escherichia coli* following protocols described previously [12, 20]. Briefly, DNA sequence data for murine collagen VII was retrieved from GenBank using the accession number NM_007738 [21]. Primers for polymerase chain reactions (PCR) were synthesized by MWG Biotech (Ebersberg, Germany; Table 1). The cDNA fragments were obtained by PCR on a cDNA pool generated by reverse transcription of mRNA extracted from cultured PAM212 cells or on synthetic DNA encoding for fragments of collagen VII provided by GenScript (Piscataway, NJ, USA). Restriction sites for *Bam*HI and *Sal*I were introduced by primers (Table 1).

**Table 1** Primer sequences for PCR amplification of cDNA fragments of murine collagen VII

| Fragment | Size (bp) | Primer sequences (5′–3′) |
|---|---|---|
| mCVII-1 | 851 | F: **GATCGGATCCCAGCCCAGAGATAGAGTGACCTGCAC** |
| | | R: **GATCGTCGACTCATGGCCGGAGACCCTGGAG** |
| mCVII-2 | 957 | F: **GATCGGATCCCGACAGGAGGTGAACATC** |
| | | R: **GATCGTCGACTCATCTGTGGATCGTTAGGATG** |
| mCVII-3 | 972 | F: **GATCGGATCCTTTGACTTAGATGATGTTCG** |
| | | R: **GATCGTCGACTCAGCTCTGCACAACTTGAAG** |
| mCVII-4 | 788 | F: **GATCGGATCCCTGGAGACTCTTCAAGTTG** |
| | | R: **GATCGTCGACTCAGATGTCACGGATCTTTCG** |
| mCVII-5 | 671 | F: **GATCGGATCCCCACTGAATAGTTCCCATG** |
| | | R: **GATCGTCGACTCACTTAATGCCAGGAGACCC** |
| mCVIICr | 698 | F: **GATCGGATCCACCCACGTAGCTGGTGTGGATG** |
| | | R: **GATCGTCGACTCACACTGGGGTCCAGGTCAAAG** |
| mCOL-Z | 491 | F: **GATCGGATCCTGCCAGGGCCAGTTTATTG** |
| | | R: **GATCGTCGACATCATCCGGGCCTCAGTCCTAG** |

*F* Forward primer, *R* reverse primer

Collagen VII cDNA fragments were cloned into linearized pGEX-6P-1 (Amersham Biosciences, Freiburg, Germany), resulting in the recombinant vectors pGEX-mCOL7-1, pGEX-mCOL7-2, pGEX-mCOL7-3, pGEX-mCOL7-4, pGEX-mCOL7-5, pGEX-mCOL7Cr, and pGEX-mCOL7-Z. For the generation of recombinant murine collagen VII with a His-tag, the DNA fragments of collagen VII were subcloned in pQE41 (Amersham Biosciences), resulting in the recombinant vectors pQE41-mCOL7-1, pQE41-mCOL7-2, pQE41-mCOL7-3, pQE41-mCOL7-4, pQE41-mCOL7-5, pQE41-mCOL7Cr, and pQE41-mCOL7-Z. Correct ligation and in-frame insertion of the various DNA fragments were confirmed by DNA sequence analysis. Recombinant GST fusion and His-tagged proteins were expressed in *E. coli* TOP 10 and XL1-Blue and purified by gluthatione agarose and metallochelate affinity chromatography, respectively as described [22, 23].

IF microscopy and immunoblot analysis

Detection of serum autoantibodies followed published protocols with minor modifications [22, 24]. Briefly, after incubating with diluted serum, the frozen sections of murine and human skin were treated with 100-fold diluted AlexaFluor 488 conjugated Abs to human IgG (Invitrogen). The rabbit polyclonal antibody SA6310 was produced as described previously [5], against the recombinant GST-fusion protein containing a sequence of murine collagen VII (GST-mCVIICr) generated for this study. IgG subclass detection in the sera of patients was done following previously described protocols [14]. Briefly, frozen sections of human salt-split skin were incubated in a first step with 10-fold diluted EBA sera, and in a second step with 500-fold diluted, biotin-conjugated monoclonal mouse Abs specific to human IgG1, IgG2, IgG3 and IgG4 (clones HP6070, HP6014, HP6047, HP6023; Invitrogen). As a tertiary detecting reagent, we have used an Alexa-Fluor 488-conjugated Streptavidin. Extracts of murine dermis were prepared as described [5]. Recombinant proteins were fractionated by 12% SDS-PAGE, transferred to nitrocellulose and analyzed by immunoblotting [5].

Measurement of autoantibody levels by ELISA

ELISA using recombinant murine collagen VII was performed at room temperature in 96-well microtiter plates. The optimal working conditions of the assay were defined by chessboard titrations with dilutions of Ag and secondary Ab, as described [14]. The optimized ELISA was run under the following conditions: wells were coated with equimolar amounts of purified His-mCVII fragments in 0.1 M bicarbonate buffer (pH 9.6). After blocking, wells were incubated for 1 h with a 100-fold dilution of patients' sera previously preadsorbed with cleared cell-lysate of bacteria transformed with pQE41 wildtype vector. Bound Abs were detected using a 5,000-fold dilution of an HRP-labeled rabbit anti-mouse IgG Ab (Abcam) and orthophenylene diamine (Dako).

Complement-fixation assay

Complement-fixing activity of EBA patients' autoantibodies to the dermal–epidermal junction was determined using a modification of published protocols [25]. Briefly, cryosections of human and murine skin were incubated with EBA patient serum or normal human serum obtained from healthy donors at 37°C for 30 min followed by washing with PBS pH 7.2, twice for 10 min. Subsequently, sections were treated with fresh human serum from healthy donors as a source of mammalian complement,

diluted 1:5 with Gelatin Veronal Buffer (Sigma), for 30 min at 37°C. Complement deposition was visualized with a monoclonal antibody to human C3 (Acris Antibodies) and an AlexaFluor-488-conjugated anti-mouse IgG antibody (Invitrogen).

### Ex vivo cryosection assay

The ability of EBA patients' autoantibodies to activate human leukocytes was assessed using an ex vivo assay of antibody-induced granulocyte-dependent dermal–epidermal separation in cryosections of normal human and murine skin, respectively, as described previously [25, 26].

### Sequence analysis

Protein sequences of murine and human collagen VII were retrieved from Genbank. Sequence alignments were performed using the algorithm developed by Smith and Waterman [27]. The antigenic determinants of collagen VII were analyzed by both MIF Bioinformatic Tools (http://bio.dfci.harvard.edu/Tools/antigenic.html) and the Antigenic routine of the EMBOSS package, which computes an antigenic score based on a prediction algorithm developed by Kolaskar and Tongaonkar [28].

## Results

### In silico analysis of antigenic epitopes of human and murine collagen VII

The murine and human forms of pro-collagen VII both with a length of 2,944 aa show by analysis using the Smith–Waterman algorithm an identity and similarity of 84.1 and 89.2%, respectively (see detailed results in ESM). B cell epitopes of murine and human collagen VII only partly overlap as predicted by analysis using specialized bioinformatic software (ESM, Table 1).

### Analysis of cross-reactivity of EBA sera by IF microscopy on murine skin sections

The reactivity of IgG autoantibodies from serum of 35 patients with EBA was analyzed by IF microscopy using human and mouse skin sections. The results of this analysis are summarized in Table 2 and representative examples are shown in Fig. 1. Twenty-eight of 30 EBA sera, which reacted with the dermal–epidermal junction of the human skin, also recognized the murine epidermal basement membrane zone.

### Generation of recombinant proteins

The cDNA sequences coding for seven fragments of murine collagen VII were cloned into prokaryotic expression vectors and expressed in *Escherichia coli* (Table 1; Fig. 2). The GST fusion and His-tagged murine collagen VII fragments were purified by glutathione- and metallochelate-affinity chromatography, respectively. All proteins migrated consistently with their calculated masses when separated by SDS-PAGE (ESM, Fig. 1).

### Molecular analysis of cross-reactivity of EBA sera with murine collagen VII

To characterize the molecular target(s) of EBA autoantibodies in murine skin, the reactivity of EBA sera was analyzed with the recombinant His-tagged fragments of murine collagen VII by ELISA and immunoblotting. The results of these analyses are summarized in Table 2 and representative examples are shown in Figs. 3 and 4. IgG autoantibodies from 14 of 19 EBA patients recognized recombinant murine collagen VII by immunoblotting.

### Mapping of epitopes on murine collagen VII recognized by EBA sera

Autoantibodies from EBA patients bind to several epitopes within the NC1 and NC2 domains of human collagen VII [10, 11]. The distribution of antigenic determinants recognized by EBA autoantibodies in murine collagen VII molecule was analyzed by immunoblotting and ELISA with different fragments of murine collagen VII (Table 2; Fig. 4). IgG autoantibodies from 5, 4, 5, 7, 4, and none of 19 EBA patients' sera recognized fragments 1, 2, 3, 4, 5, and Z, respectively.

### EBA autoantibodies fix complement to the murine dermal–epidermal junction

To address the question whether EBA autoantibody binding to the dermal–epidermal junction of murine skin can activate complement locally, their complement-binding ability was assessed ex vivo by an immunofluorescent complement-fixation test. The results of this assay are summarized in Fig. 5. In contrast to sera from healthy donors (Fig. 5a, b), autoantibodies from 6 of 11 EBA patients showing complement-binding potential in human skin sections (Fig. 5c), also fixed complement at the dermal–epidermal junction of murine skin (Fig. 5d).

**Table 2** Cross-reactivity of EBA sera with murine collagen VII

| Patient | Age (years) | Reactivity with | | | | | | | | CBT IF scores[a] | | DES | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Human skin (IIF)[a] | Murine skin (IIF)[a] | mCVII fragments (ELISA/IB) | | | | | | | Human skin | Mouse skin | Human skin | Mouse skin |
| | | | | 1 | 2 | 3 | 4 | 5 | Cr | Z | | | | |
| EBA1 | 51 | ++++ | ++++ | X | | X | | | | | ++++ | ++ | ++++ | ++++ |
| EBA2 | 19 | ++ | ++ | | | | X | X | X | | − | − | − | − |
| EBA3 | 20 | ++++ | +++ | X | | | X | X | | | ++ | ++ | ++ | ++ |
| EBA4 | 13 | ++ | + | | | | X | | | | n.d. | n.d. | n.d. | n.d. |
| EBA5 | 46 | ++ | + | | X | X | | | | | n.d. | n.d. | n.d. | n.d. |
| EBA6 | 67 | + | + | − | − | − | − | − | − | − | n.d. | n.d. | + | − |
| EBA7 | 77 | + | + | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| EBA8 | − | ++ | ++ | | X | X | | | | | n.d. | n.d. | +++ | − |
| EBA9 | 59 | +++ | +++ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | +++ | + | ++ | − |
| EBA10 | 56 | ++ | ++ | | | | X | | X | | n.d. | n.d. | +++ | ++ |
| EBA11 | 89 | ++ | ++ | | | X | X | | | | n.d. | n.d. | ++ | + |
| EBA12 | 49 | + | + | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| EBA13 | − | ++++ | ++++ | − | − | − | − | − | − | − | +++ | − | ++++ | +++ |
| EBA14 | 66 | ++ | ++ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | + | + |
| EBA15 | − | +++ | +++ | X | | | | | | | − | − | ++++ | − |
| EBA16 | 71 | +++ | +++ | − | − | − | − | − | − | − | − | − | ++ | ++ |
| EBA17 | 60 | +++ | +++ | | X | | | | | | ++ | ++ | +++ | − |
| EBA18 | 49 | ++++ | +++ | − | − | − | − | − | − | − | ± | − | ++ | − |
| EBA19 | 26 | +++ | ++ | X | | | X | | | | n.d. | n.d. | +++ | − |
| EBA20 | 68 | + | + | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| EBA21 | 40 | ++++ | ++++ | | X | X | | X | | | + | ± | ++++ | − |
| EBA22 | 75 | − | − | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| EBA23 | 61 | + | + | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| EBA24 | 85 | ++ | ++ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | − | − | + | − |
| EBA25 | − | − | − | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| EBA26 | 57 | ++ | ++ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | − | − |
| EBA27 | 49 | + | − | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| EBA28 | − | +++ | +++ | | | | X | X | X | | ++ | − | ++ | − |
| EBA29 | − | +++ | ++ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | ++++ | ± | +++ | ++ |
| EBA30 | − | ++++ | +++ | − | − | − | − | − | − | − | ± | − | ++ | ++ |
| EBA31 | 69 | ++++ | +++ | X | | | | | | | ± | − | ++ | + |
| EBA32 | 80 | − | − | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| EBA33 | 22 | ± | − | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| EBA34 | 7 | − | − | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| EBA35 | − | − | − | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| TOTAL | | 30/35 | 28/35 | 5/19 | 4/19 | 5/19 | 7/19 | 4/19 | 3/19 | 0/19 | 11/15 | 6/15 | 20/22 | 10/22 |

[a] The fluorescence intensity has been scored as follows: − no staining, + focal faint staining of the basal membrane, ++ faint staining of the basal membrane, +++ medium staining of the basal membrane, ++++ strong staining of the basal membrane

### EBA sera induce dermal–epidermal separation of murine skin cryosections

Autoantibodies against collagen VII have been shown to recruit and activate granulocytes to the dermal–epidermal junction and induce subepidermal splits in cryosections of human skin when coincubated with granulocytes from healthy donors [12, 17, 29]. To assess the ability of EBA patient autoantibodies to induce granulocyte-dependent dermal–epidermal separation in murine skin, sera from EBA patients and healthy donors were incubated with murine and human skin cryosections. Representative examples of this assay are shown in Fig. 6. In contrast to the normal human sera (Fig. 6a, b), after the addition of

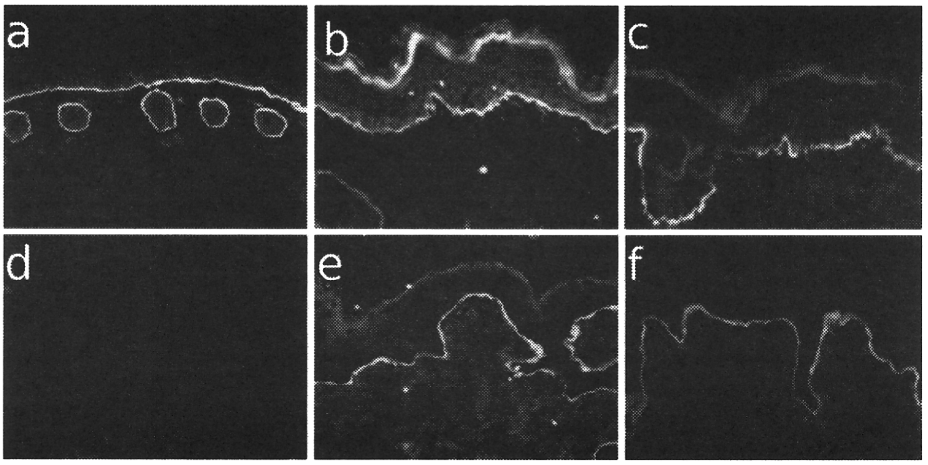


**Fig. 1** Cross-reactivity of EBA patients sera with murine skin by immunofluorescence microscopy. Cryosections of **a–d** murine and **e**, **f** human skin were incubated with **a** serum from a rabbit immunized with GST-mCVIICr (SA6310), **b**,**e** serum from EBA3, **c**,**f** serum from EBA1, and **d** normal human serum (NHS). After washing in PBS, sections were incubated with **a** anti-rabbit IgG and **b–f** anti-human IgG both labeled with AlexaFluor 488 (magnifications, all ×200)

human leukocytes, autoantibodies from 20 and 10 EBA patients induced subepidermal splits in the cryosections of (Fig. 6c) human and (Fig. 6d) murine skin, respectively.

## Discussion

Several animal models of blister formation by the passive transfer of collagen VII-specific antibodies into mice have been recently established [5, 13, 30]. In addition to demonstrating the autoimmune nature of EBA, these models represent exquisite tools for studying the cellular and molecular aspects of the autoantibody-induced tissue damage [5, 16, 17, 23, 25]. However, among other limitations, the epitopes recognized by patient autoantibodies may not match when murine collagen VII is compared with its human ortholog. Previous studies and our present analysis indeed show a relatively low degree of homology between human and murine collagen VII compared with the homology of other collagen molecules [31]. In addition, a cross-reactivity of autoantibodies with murine skin could not be previously documented in all tested EBA patients [5]. Furthermore, as our present in silico analysis shows, the predicted B cell epitopes on murine and human collagen VII only partly overlap. Despite these possible limitations, patient autoantibodies are in theory ideal reagents in terms of both specificity and effector functions to use for the study of blister formation. Therefore, in the present work, we characterized the epitopes on murine collagen VII recognized by EBA patient autoantibodies.

In a first set of experiments, we analyzed the reactivity of serum autoantibodies with the dermal–epidermal junction in a large group of EBA patients. In line with previous data [5], the results show a cross-reactivity of EBA autoantibodies with murine skin in the majority of our EBA

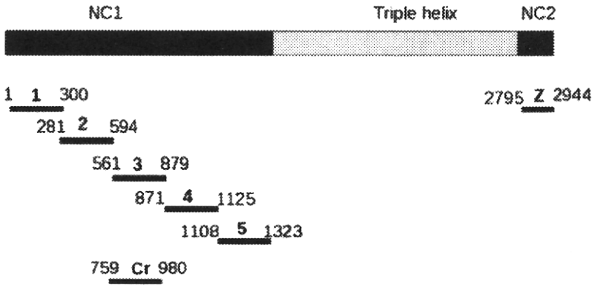


**Fig. 2** Recombinant fragments of collagen VII generated in this study. Collagen VII is composed of three identical α chains, each consisting of a central triple helical collagenous domain, flanked by a large amino-terminal noncollagenous domain (*NC1*) and a smaller carboxy-terminal noncollagenous domain (*NC2*). Fragments of murine collagen VII cDNA corresponding to the NC1 and NC2 domains were cloned into pGEX-6P-1 and pQE41 and expressed in *E. coli*. Amino acid residue numbers are shown above the fragments

patients. As expected, these EBA sera specifically reacted with recombinant murine collagen VII as demonstrated by immunoblot analysis.

To map the epitopes on murine collagen VII recognized by the EBA sera, we used overlapping recombinant fragments covering the NC1 and NC2 domains of this antigen. The overall pattern of reactivity is matching the one reported for the reactivity with the human collagen VII fragments [10, 11, 32]. We show that autoantibodies in EBA sera react with different epitopes within the NC1, but not NC2, domains of murine collagen VII.

In further experiments, we addressed the pathogenic relevance of autoantibodies from a relatively large number of EBA patients cross-reacting with murine skin. Because of the limited amounts of EBA serum, we used two ex vivo assays to assess the effector functions of autoantibodies. The capacity of autoantibodies to fix complement C3 at the murine dermal–epidermal junction was reduced compared to their ability to activate complement on human skin sections. This finding is compatible with the

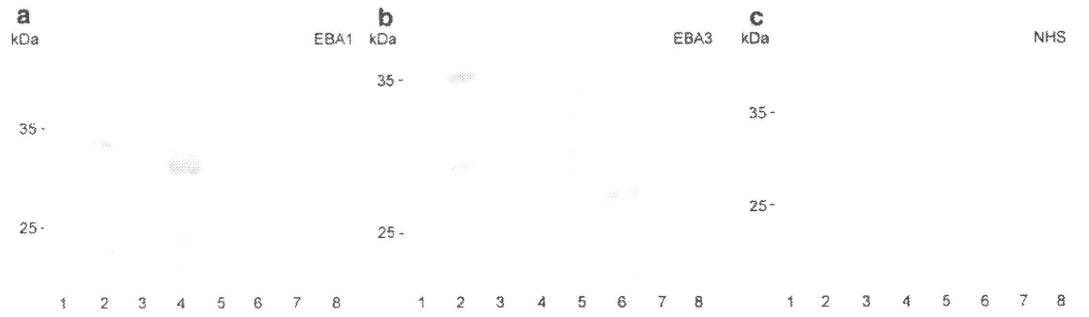


**Fig. 3** Immunoblot analysis of the EBA sera reactivity with mCVII. His-tagged recombinant mCVII fragments 1, 2, 3, 4, 5 and Z (*lanes 2–8*) as well as His-DHFR (*lane 1*) were separated by 12% SDS-PAGE and electrophoretically transferred to nitrocellulose. The membranes were immunoblotted with **a** serum from patients EBA1, **b** EBA3, and **c** normal human serum (*NHS*). Migration positions of molecular weight markers (kDa) are shown on the *left*

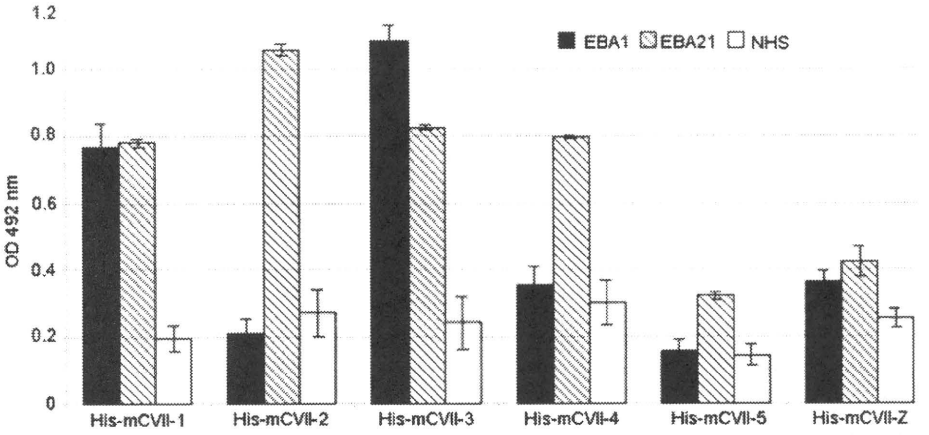


**Fig. 4** Analysis of the reactivity of EBA sera with murine collagen VII by ELISA. Levels of serum IgG autoantibodies were measured in triplicate by ELISA using recombinant fragments of murine collagen VII as described in "Materials and methods". OD readings of serum samples from patients EBA1, EBA21 and from a healthy donor are given as mean ± SD

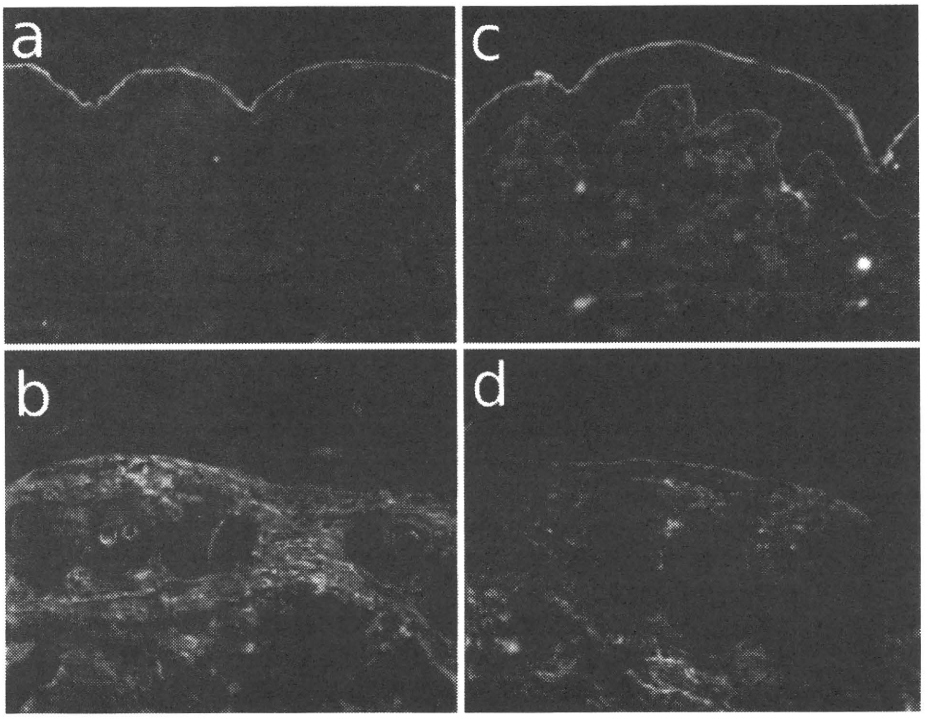


**Fig. 5** EBA patient autoantibodies fix complement to the dermal–epidermal junction of murine skin sections. Frozen sections of human (**a**,**c**) and murine (**b**,**d**) skin were incubated with serum from a healthy donor (**a**,**b**) and from EBA1 patient (**c**,**d**). After washing in phosphate-buffered saline, the sections were further incubated with fresh human serum as a source of complement. C3 deposits were visualized by incubation with a monoclonal antibody specific to human C3 followed by an AlexaFluor-488-conjugated anti-mouse IgG antibody. While normal human serum (**a**,**b**) did not fix complement in the cryosections, the EBA serum bound complement at the dermal–epidermal junction of both human (**c**) and murine (**d**) skin sections

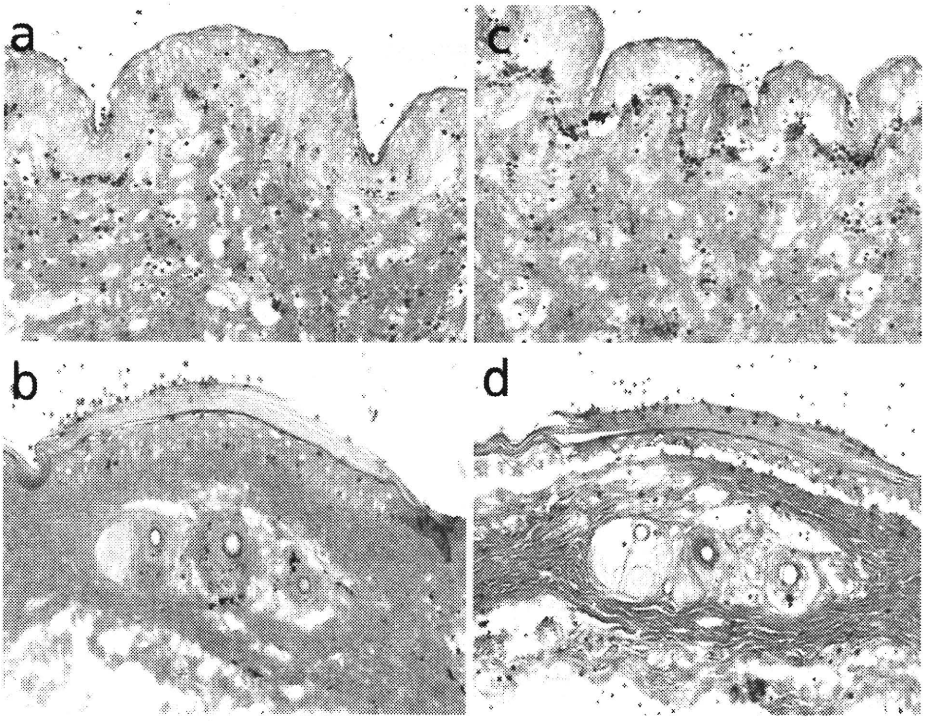


**Fig. 6** EBA patient autoantibodies induce dermal–epidermal separation in sections of murine skin. Frozen sections of human (**a**,**c**) and murine (**b**,**d**) skin were incubated with serum from a healthy donor (**a**,**b**) and from EBA1 patient (**c**,**d**). After washing in phosphate-buffered saline, the sections were further incubated with fresh human serum as a source of leucocytes. In contrast to sections incubated with serum from a healthy donor (**a**,**b**), sections treated with serum from the EBA patient (**c**,**d**) developed dermal–epidermal separation after incubation with normal human granulocytes

assumption that EBA autoantibodies recognize fewer (pathogenic) epitopes on murine collagen VII compared to its human ortholog. Importantly, autoantibodies from a subgroup of the EBA sera which induced dermal–epidermal separation in human skin were also pathogenic in the murine skin. In line with our previous observation that complement activation is not required for the granulocyte-dependent dermal–epidermal separation in this model [22], the ability of EBA autoantibodies to induce subepidermal splits did not closely match their complement-fixing properties. In EBA patients, the autoantibodies against collagen VII belong to different IgG subclasses and their complement-fixing ability does not correlate with the inflammatory or classical clinical forms of the disease [33]. However, since results from in vivo models of EBA demonstrate that complement activation is a prerequisite for the antibody-induced blistering and suggest that IgG subclass of autoantibodies is determining their pathogenicity, further investigation of the association of complement-binding capacity and IgG subclass of autoantibodies with disease activity in EBA patients is required [14, 16].

One major disadvantage of using IgG from EBA patients for in vivo studies is their low availability [18]. This issue could be addressed by generating human autoantibodies recombinantly or by classical hybridoma technology in quantities sufficient to sustain long-term research using this model. Our present results provide the rationale and the tools to develop recombinant or monoclonal human autoantibodies specific to collagen VII. The characterization of the pathogenic epitopes recognized by patient autoantibodies is also relevant for addressing further questions, including the epitope-specific affinity purification of patient serum autoantibodies, the development of an improved animal model by peptide immunization, and the induction of immunological tolerance.

In conclusion, our results demonstrate for the first time that blister-inducing EBA autoantibodies recognize multiple epitopes on murine collagen VII. Patient autoantibodies binding to murine collagen VII epitopes retain in part their pathogenic activity. Our results significantly add to the rationale of studying the blister formation in EBA by passively transferring patients' autoantibodies and suggest the existence of multiple pathogenic epitopes in EBA. In addition, the reagents developed in this work should be useful for pathogenetic studies and the development of more specific therapeutic approaches.

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## References


1. Nishie W, Sawamura D, Goto M, Ito K, Shibaki A, McMillan J, Sakai K, Nakamura H, Olasz E, Yancey K, Akiyama M, Shimizu H (2007) Humanization of autoantigen. Nat Med 13:378–383
2. Toyka KV, Brachman DB, Pestronk A, Kao I (1975) Myasthenia gravis: passive transfer from man to mouse. Science 190:397–399
3. Liu Z, Diaz LA, Troy JL, Taylor AF, Emery DJ, Fairley JA, Giudice GJ (1993) A passive transfer model of the organ-specific autoimmune disease, bullous pemphigoid, using antibodies generated against the hemidesmosomal antigen, BP180. J Clin Invest 92:2480–2488
4. Lazarova Z, Yee C, Darling T, Briggaman RA, Yancey KB (1996) Passive transfer of anti-laminin 5 antibodies induces subepidermal blisters in neonatal mice. J Clin Invest 98:1509–1518
5. Sitaru C, Mihai S, Otto C, Chiriac MT, Hausser I, Dotterweich B, Saito H, Rose C, Ishiko A, Zillikens D (2005) Induction of dermal–epidermal separation in mice by passive transfer of antibodies specific to type VII collagen. J Clin Invest 115:870–878
6. Anhalt GJ, Labib RS, Voorhees JJ, Beals TF, Diaz LA (1982) Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. N Engl J Med 306:1189–1196
7. Sitaru C (2009) Bullous pemphigoid: a prototypical antibody-mediated organ-specific autoimmune disease. J Invest Dermatol 129:822–824
8. Mihai S, Sitaru C (2007) Immunopathology and molecular diagnosis of autoimmune bullous diseases. J Cell Mol Med 11:462–481
9. Sitaru C, Zillikens D (2005) Mechanisms of blister induction by autoantibodies. Exp Dermatol 14:861–875
10. Lapiere JC, Woodley DT, Parente MG, Iwasaki T, Wynn KC, Christiano AM, Uitto J (1993) Epitope mapping of type VII collagen. Identification of discrete peptide sequences recognized by sera from patients with acquired epidermolysis bullosa. J Clin Invest 92:1831–1839
11. Ishii N, Yoshida M, Hisamatsu Y, Ishida-Yamamoto A, Nakane H, Iizuka H, Tanaka T, Hashimoto T (2004) Epidermolysis bullosa acquisita sera react with distinct epitopes on the NC1 and NC2 domains of type VII collagen: study using immunoblotting of domain-specific recombinant proteins and postembedding immunoelectron microscopy. Br J Dermatol 150:843–851
12. Sitaru C, Kromminga A, Hashimoto T, Bröcker EB, Zillikens D (2002) Autoantibodies to type VII collagen mediate Fcgamma-dependent neutrophil activation and induce dermal–epidermal separation in cryosections of human skin. Am J Pathol 161:301–311
13. Woodley DT, Ram R, Doostan A, Bandyopadhyay P, Huang Y, Remington J, Hou Y, Keene DR, Liu Z, Chen M (2006) Induction of epidermolysis bullosa acquisita in mice by passive transfer of autoantibodies from patients. J Invest Dermatol 126:1323–1330
14. Sitaru C, Chiriac MT, Mihai S, Büning J, Gebert A, Ishiko A, Zillikens D (2006) Induction of complement-fixing autoantibodies against type VII collagen results in subepidermal blistering in mice. J Immunol 177:3461–3468
15. Chen M, Doostan A, Bandyopadhyay P, Remington J, Wang X, Hou Y, Liu Z, Woodley DT (2007) The cartilage matrix protein subdomain of type VII collagen is pathogenic for epidermolysis bullosa acquisita. Am J Pathol 170:2009–2018
16. Mihai S, Chiriac MT, Takahashi K, Thurman JM, Holers VM, Zillikens D, Botto M, Sitaru C (2007) The alternative pathway of complement activation is critical for blister induction in experimental epidermolysis bullosa acquisita. J Immunol 178:6514–6521
17. Chiriac MT, Roesler J, Sindrilaru A, Scharffetter-Kochanek K, Zillikens D, Sitaru C (2007) NADPH oxidase is required for neutrophil-dependent autoantibody-induced tissue damage. J Pathol 212:56–65
18. Sitaru C (2007) Experimental models of epidermolysis bullosa acquisita. Exp Dermatol 16:520–531
19. Yuspa SH, Hawley-Nelson P, Koehler B, Stanley JR (1980) A survey of transformation markers in differentiating epidermal cell lines in culture. Cancer Res 40:4694–4703
20. Olaru F, Mihai S, Petrescu I, Zillikens D, Sitaru C (2006) Generation and characterization of monoclonal antibodies against the intracellular domain of human type XVII collagen. Hybridoma 25:158–162
21. Li K, Tamai K, Tan EM, Uitto J (1993) Cloning of type XVII collagen. Complementary and genomic DNA sequences of mouse 180-kilodalton bullous pemphigoid antigen (BPAG2) predict an interrupted collagenous domain, a transmembrane segment, and unusual features in the 5′-end of the gene and the 3′-untranslated region of the mRNA. J Biol Chem 268:8825–8834
22. Sitaru C, Schmidt E, Petermann S, Munteanu LS, Bröcker E, Zillikens D (2002) Autoantibodies to bullous pemphigoid antigen 180 induce dermal–epidermal separation in cryosections of human skin. J Invest Dermatol 118:664–671
23. Sesarman A, Sitaru AG, Olaru F, Zillikens D, Sitaru C (2008) Neonatal Fc receptor deficiency protects from tissue injury in experimental epidermolysis bullosa acquisita. J Mol Med 86:951–959
24. Sitaru C, Powell J, Messer G, Bröcker E, Wojnarowska F, Zillikens D (2004) Immunoblotting and enzyme-linked immunosorbent assay for the diagnosis of pemphigoid gestationis. Obstet Gynecol 103:757–763
25. Sesarman A, Mihai S, Chiriac MT, Olaru F, Sitaru AG, Thurman JM, Zillikens D, Sitaru C (2008) Binding of avian IgY to type VII collagen does not activate complement and leucocytes and fails to induce subepidermal blistering in mice. Br J Dermatol 158:463–471
26. Mihai S, Chiriac MT, Herrero-González JE, Goodall M, Jefferis R, Savage COS, Zillikens D, Sitaru C (2007) IgG4 autoantibodies induce dermal–epidermal separation. J Cell Mol Med 11:1117–1128
27. Smith TF, Waterman MS (1981) Identification of common molecular subsequences. J Mol Biol 147:195–197
28. Kolaskar A, Tongaonkar P (1990) A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett 276:172–174
29. Shimanovich I, Mihai S, Oostingh GJ, Ilenchuk TT, Bröcker E, Opdenakker G, Zillikens D, Sitaru C (2004) Granulocyte-derived elastase and gelatinase B are required for dermal–epidermal separation induced by autoantibodies from patients with epidermolysis bullosa acquisita and bullous pemphigoid. J Pathol 204:519–527
30. Woodley DT, Chang C, Saadat P, Ram R, Liu Z, Chen M (2005) Evidence that anti-type VII collagen antibodies are pathogenic and responsible for the clinical, histological, and immunological features of epidermolysis bullosa acquisita. J Invest Dermatol 124:958–964
31. Li K, Christiano AM, Copeland NG, Gilbert DJ, Chu ML, Jenkins NA, Uitto J (1993) cDNA cloning and chromosomal mapping of the mouse type VII collagen gene (Col7a1): evidence for rapid evolutionary divergence of the gene. Genomics 16:733–739
32. Tanaka T, Furukawa F, Imamura S (1994) Epitope mapping for epidermolysis bullosa acquisita autoantibody by molecularly cloned cDNA for type VII collagen. J Invest Dermatol 102:706–709
33. Gandhi K, Chen M, Aasi S, Lapiere JC, Woodley DT, Chan LS (2000) Autoantibodies to type VII collagen have heterogeneous subclass and light chain compositions and their complement-activating capacities do not correlate with the inflammatory clinical phenotype. J Clin Immunol 20:416–423

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# Novel ELISA systems for antibodies to desmoglein 1 and 3: correlation of disease activity with serum autoantibody levels in individual pemphigus patients

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**Abstract:** Pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are intraepidermal blistering skin diseases. PV is characterised by autoantibodies directed against desmoglein (Dsg) 3 and in patients with the mucocutaneous variant also against Dsg 1, whereas in PF, only Dsg 1 is targeted. Here, ectodomains of Dsg 3 and Dsg 1 were recombinantly expressed in a human cell line (HEK293) and applied as authentic solid phases in ELISA test systems. Autoantibodies against Dsg 3 and/or Dsg 1 could be detected in 71 (100%) of 71 PV sera and against Dsg 1 in 48 (96%) of 50 PF sera. Control sera showed reactivity with Dsg 3 and Dsg 1 in 0.2% and 0.7%, respectively, of 401 healthy blood donors and in 2.1% of 48 randomly selected patients with bullous pemphigoid. No reactivity with Dsg 1 and 3 was detected in 21 patients with linear IgA disease. For both pemphigus variants, a statistically significant correlation between clinical severity and autoantibody levels was observed as demonstrated for 10 PV and 5 PF patients. In conclusion, the use of the ectodomains of Dsg 3 and 1 as target antigens expressed in a human cell line resulted in sensitive and specific ELISA systems for both diagnosis and monitoring of PV and PF.

**Key words:** autoimmunity – detection – disease activity – ELISA

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## Introduction

Pemphigus is a group of severe autoimmune blistering skin diseases characterised by circulating IgG autoantibodies to different keratinocyte cell surface proteins (1). Most evidence has been accumulated on the pathogenic relevance of autoantibodies directed to the cell-cell adhesion molecules desmoglein (Dsg) 3 and 1 (1). Antibodies against different other antigens, including cholinergic receptors and annexins, have also been implicated in the pathogenesis of this disease (2). Pemphigus vulgaris (PV) and pemphigus foliaceus (PF) represent the two most frequent pemphigus subtypes.

Diagnosis of pemphigus is based on the clinical appearance and the detection of tissue-bound and serum autoantibodies. Direct immunofluorescence (IF) microscopy of a perilesional biopsy is still the diagnostic gold standard and typically reveals an intercellular deposition of IgG in the epidermis/epithelium. Serum analysis had previously relied on monkey or guinea pig oesophagus as most sensitive substrates for indirect IF microscopy that detect circulating autoantibodies in about 90% of pemphigus patients (3). For further specification of the circulating antibodies in pemphigus, immunoblotting with various cell-based extracts and recombinant fragments of Dsg 3 and 1 has been applied. However, immunoblotting has been proven inadequate since the majority of epitopes on Dsg 3 and 1 appear to be conformational (4–11). Subsequently, several ELISA systems have been developed using extracts of bovine skin and recombinant portions of Dsg 3 and 1 expressed in *Escherichia coli* or Sf21 insect cells (7,12–15). Two ELISA systems based on the insect cell-expressed



 



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recombinant ectodomains of Dsg 3 and 1 were shown to be superior to indirect IF microscopy due to less interpreter dependency (12,16,17) and have therefore greatly improved the standardisation between different laboratories.

In the present study, the ectodomains of Dsg 3 and 1 were expressed in the human cell line HEK293 to allow authentic protein folding as well as post-translational modification. They were applied in two novel, specific and sensitive ELISA systems which were shown to be most suitable for both serological diagnosis of PV and PF and monitoring disease activity during the course of the disease.

## Materials and methods

### Patients

Sera from 71 patients with PV (23 males, 48 females; mean age, 60 years; range, 20–98 years) and 50 patients with PF (26 males, 24 females; mean age, 60 years; range, 17–94 years) were collected from three different departments of dermatology: University of Lübeck, Germany, University of Würzburg, Germany, and University of Kurume Medical School, Japan. Pemphigus sera were obtained from patients who fulfilled the following criteria: (i) active disease with mucous membrane lesions and/or blisters/erosions on the skin in PV as well as blisters/scaly erosions on the skin in PF at the time of serum collection, (ii) intercellular binding of IgG and/or C3 in the epidermis of a perilesional biopsy by direct IF microscopy, and (iii) serum autoantibodies that reacted with the epithelium of monkey oesophagus by indirect IF microscopy and/or with Dsg 3 (in PV) or Dsg 1 (in PF) by ELISA (MBL, Nagoya, Japan). In addition, sera from patients with bullous pemphigoid (BP; $n = 48$) and linear IgA dermatosis (LAD; $n = 21$) were included. BP/LAD were confirmed by (i) typical clinical presentations, (ii) subepidermal split formation by a lesional histopathology, (iii) linear binding of IgG or C3/IgA at the dermal-epidermal junction by direct IF microscopy, and (iv) serum IgG antibodies to BP180 by ELISA (EUROIMMUN, Lübeck, Germany) and serum IgA reactivity against the cell-derived ectodomain of BP180 (LAD-1) by Western blotting of keratinocyte extract, respectively. Sera from healthy blood donors (HBD; $n = 401$) served as additional controls. All sera were stored at −20°C until assayed.

In 15 patients (PV, $n = 10$; PF, $n = 5$), sera were analysed during the course of the disease at at least three different time points. In five of the 10 PV patients, lesions were restricted to the mucosa, in the other five patients, the mucocutaneous variant was present. Disease activity for skin lesions: score of 3, >10 lesions; score of 2, 4–10 lesions; score of 1, 1–3 lesions; score of 0, no lesions. The disease score for mucosal lesions was adopted from the pemphigus disease area index (PDAI) (18) with minor modifications: score of 3, >3 lesions; score of 2, 2–3 lesions; score of 1, 1 lesion; score of 0, no lesions. A single lesion was defined as area with blister, erosion, or erythema with positive Nikolski sign; lesions needed to be separated by intact epithelium. Scoring was done retrospectively based on photographs and patient records; serum was drawn on the same day when photographs were taken.

### Cloning of vector constructs coding for Dsg 3(ec)-His and Dsg 1(ec)-His

The cloning strategy is detailed in Table 1. The final cDNAs were cleaved with NcoI/Esp3I (Dsg 3 fragment 1), Esp3I/XhoI (Dsg 3 fragment 2), and NcoI/SalI (Dsg 1), respectively. A mixture of both cDNAs coding for Dsg 3 and the cDNA coding for Dsg 1 were then ligated individually with pTriEx1 (Merck Biosciences, Darmstadt, Germany) linearized with NcoI/XhoI, resulting in pTriEx1-Dsg 3(ec)-His and pTriEx1-Dsg 1(ec)-His. The correctness of

**Table 1.** Primer sequences for PCR amplification of cDNA fragments of desmoglein (Dsg) 3 and 1.

| Protein | Restriction sites | Primer sequences (5′ → 3′) |
|---|---|---|
| Dsg 3 | NcoI | F1: ATACCATGGGGCTCTTCCCCAGAACTACAGGGGCTC |
| | Esp3I | R1: ATACGTCTCTAGATTCCAAAAGGCGGCTGATCGATTC |
| | Esp3I | F2: ATACGTCTCTATCTTTGTTGTTGACAAAAACACTGGAG |
| | XhoI | R2: ATACTCGAGCCTCCCTGAGTGCGGCCTGCCATACCTG |
| Dsg 1 | NcoI | F: ATACCATGGACTGGAGTTTCTTCAGAGTAG |
| | SalI | R: ATAGTCGACAGGACCAAAATGTACATTGTCTGATAACAAATCTTTGGCTC |

cDNAs coding for the ectodomains of Dsg 3 (accession number NM_001944) and Dsg 1 (accession number NM_001942) were produced by PCR using EST clones (Dsg 3: DKFZp781L1944Q, accession BX476267; Dsg 1: DKFZp686D19185Q, accession BX476267; Imagenes, Berlin, Germany) and DNA oligonucleotide primers indicated below. Due to a sequence variation in the Dsg 3 EST clone DKFZp781L1944Q, two individual PCR were performed (F1 + R1; F2 + R2) in order to generate a sequence according to Acc. No. NM_001944 by site directed mutagenesis.
F, forward primer; R, reverse primer.

the final constructs was confirmed by DNA sequencing (MWG, Ebersberg, Germany).

### Expression and purification of recombinant Dsg 3(ec)-His and Dsg 1(ec)-His

HEK293 cells cultured in DMEM (Invitrogen, Karlsruhe, Germany) with 10% foetal calf serum (v/v) were transfected with pTriEx1/3-Dsg 3(ec)-His using ExGen 500 (Fermentas, St. Leon-Roth, Germany). Dsg 1/3 (ec)-His was purified by immobilised metal affinity chromatography on TALON superflow (Clontech, Palo Alto, CA, USA). Protein analysis was performed by SDS-PAGE using the NuPAGE system according to the manufacturer's manual (Invitrogen) and by immunoblotting using a monoclonal antibody to hexahistidine (Merck Biosciences).

### Anti-Dsg 3 ELISA and Anti-Dsg 1 ELISA

Microtiter plates (Nunc, Langensebold, Germany) were coated with either 1.2 $\mu$g/ml Dsg 3(ec)-His or 2.5 $\mu$g/ml Dsg 1(ec)-His. Sera diluted 1:101 in PBS-0.1% (w/v) casein were incubated for 30 min before washing. As detection antibody an anti-human IgG peroxidase-conjugate (EUROIMMUN) was applied for 30 min, for visualisation tetramethylbenzidine (EUROIMMUN) for 15 min. Optical density (OD) was determined at 450 nm (reference 620 nm). All procedures were carried out at room temperature. The cut-off for positivity was validated and optimised by receiver-operating characteristics (ROC). A highly positive index patient serum was used to generate a standard curve consisting of three calibrators (2, 20, and 200 RU/ml) and covering the linear range of the assay (2–200 RU/ml; inserts of Fig. 1). RU were calculated from the OD values by this standard curve.

### Statistical analysis

ROC and prevalence analyses were performed using the SigmaPlot 10.0 analysis software (SSI, San Jose, CA, USA) and the EUROStat statistical package (EUROIMMUN). For statistical evaluation of the relation between disease score and autoantibody levels expressed by log-transformed Dsg 3 and 1 ELISA values (RU/ml), a non-parametric test procedure derived from Spearman correlation analysis was applied as provided by Gnu R statistical software (R Development Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. 2008. ISBN 3-900051-07-0) with packages 'coin' and 'lattice' was used. Spearman correlation coefficients of disease score versus log-transformed ELISA values were calculated individually for each patient. In consideration of the relatively low sample sizes the $P$ value was calculated by the use of Monte Carlo resampling with 10 000 000 replicates (package 'coin', Gnu R statistical software. A $P$-value below 0.05 was considered statistically significant.

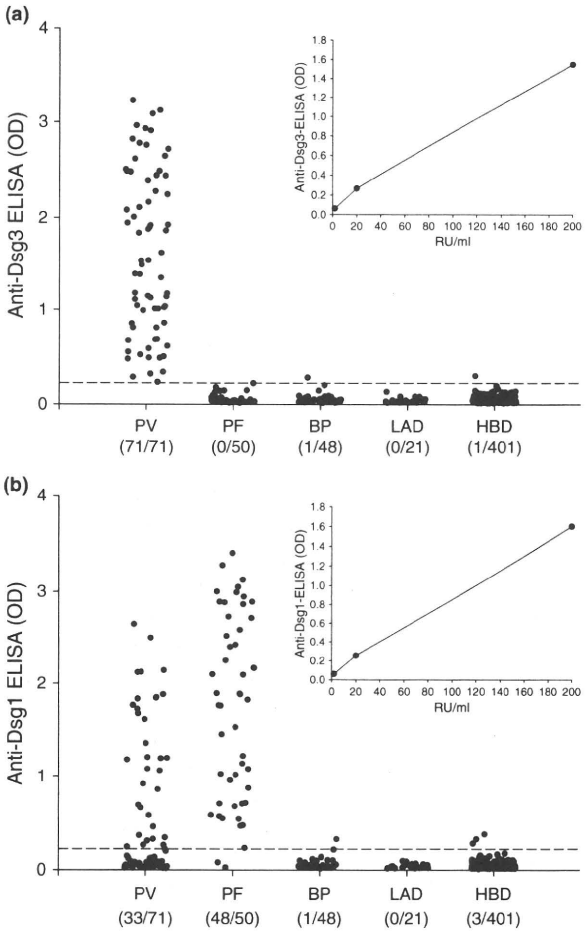


**Figure 1.** ELISA reactivities of pemphigus and control sera with the human cell-expressed ectodomains of desmoglein 3 and 1. Sera from patients with pemphigus vulgaris (PV) and pemphigus foliaceus (PF) as well as control sera from patients with bullous pemphigoid (BP), linear IgA dermatosis (LAD), and healthy blood donors (HBD) were quantitatively analysed by the novel ELISA systems based on the human cell-expressed ectodomains of desmoglein (Dsg) 3 (a) and Dsg 1 (b). Plotted data points represent optical densities (OD) measured at 450 nm. Cut-off values are indicated by dashed lines (Dsg 3, OD 0.22; Dsg 1 OD 0.21). Numbers in brackets represent positive and total cases, respectively. Typical standard curves revealed a linear range of the ELISA systems between the lower and upper detection limits of 20 and 200 RU/ml, respectively (inserts). RU, relative units.

## Results

### Eukaryotic expression and purification of the mature forms of recombinant Dsg 3 and Dsg 1

Upon expression of oligo-His-tagged ectodomains of human Dsg 3 and Dsg 1 in the human cell line HEK293, the recombinant proteins were secreted into the culture medium in their mature forms and migrated consistent



 

with their calculated masses when separated by SDS-PAGE (data not shown). Preceding experiments comparing expression of desmoglein with and without proprotein in HEK293 cells revealed the same migration positions of desmoglein 1 and 3 indicating that only the mature forms were generated (data not shown).

### Development of two ELISA systems using the human cell-expressed ectodomains of Dsg 3 and Dsg 1

ROC analysis was performed in order to determine the cut-off values of the newly developed immunoassays and to characterise their overall diagnostic performance. For this purpose, levels of autoantibodies to Dsg 3 and Dsg 1 were measured in sera from 71 PV and 50 PF patients as well as 470 controls, including 48 patients with BP, 21 with LAD, and 401 healthy blood donors. Based on a clinical specificity of ≥99%, the cut-off of both immunoassays was set at 20 RU/ml. This threshold is close to the cut-off values resulting in the calculated maximum sum of sensitivity and specificity, and it exceeds the 99th percentile of the non-diseased reference cohort. Both ELISA systems revealed high overall diagnostic performances as indicated by area under the curve (AUC) values above 0.95 and by theoretical sensitivities of 100% (anti-Dsg 3; cut-off 12.4 RU/ml) and 96% (anti-Dsg 1; cut-off 15.4 RU/ml) at a pre-defined specificity of 99% (Table 2).

### The two ELISA systems using human cell-expressed ectodomains of Dsg 3 and Dsg 1 are sensitive and specific tools for detection of pemphigus antibodies

Referring to the cut-off defined by ROC analysis, IgG reactivity to Dsg 3 was found in 71 of 71 (100%) PV patients, whereas none of the PF patients, 1 of 48 (2.1%) BP patients, none of the LAD patients, and 1 of 401 (0.2%) HBD were reactive (Fig. 1a). Using the anti-Dsg 1 ELISA, 48 of 50 (96%) PF patients were positive, as well as 1 of 48 (2.1%) BP patients, none of the LAD patients and 3 of 401 (0.7%) healthy blood donors. Among the PV patients, 33 (46.5%) had antibodies to Dsg 1 in addition to anti-Dsg 3 (Fig. 1b). According to these data, the newly developed ELISA systems revealed a sensitivity and specificity of 100% (71 of 71 PV) and 99.6% (468 of 470 controls), respectively, for anti-Dsg 3 antibodies, as well as 96% (48 of 50 PF) and 99.1% (466 of 470 controls), respectively, for anti-Dsg 1 antibodies.

The same patient sera were assayed with the anti-Dsg 3 and anti-Dsg 1 ELISA produced by MBL. Reactivity to Dsg 3 was found in all of 71 PV, 2 (4.2%) of the 48 BP, and 1 (4.8%) of the 21 LAD patients. Reactivity to Dsg 1 was detected in all of 50 PF, 2 (4.2%) of the 48 BP, and 1 (4.8%) of the 21 LAD patients sera. Consequently, these conventional ELISA systems revealed a specificity of 95.7% (negative readings in 66 of 69 controls).

**Table 2.** Characteristics of the ELISA systems using the human cell-expressed ectodomains of desmoglein (Dsg) 3 and 1 as antigenic substrates.

| Test parameters | Anti-Dsg 3 | Anti-Dsg 1 |
|---|---|---|
| AUC | 0.999 | 0.980 |
| 95% CI | 0.999–1.000 | 0.945–1.014 |
| Maximal sum of sensitivity and specificity (cut-off*) | 199.6% (18.81) | 195.2% (18.91) |
| Sensitivity at a specificity of 98% (cut-off) | 100.0% (10.0) | 96.0% (10.75) |
| Sensitivity at a specificity of 99% (cut-off) | 100.0% (12.36) | 96.0% (15.44) |
| Cut-off 98th percentile | 8.79 | 7.98 |
| Cut-off 99th percentile | 11.42 | 13.44 |

AUC, area under the curve; CI, confidence interval.
*Cut-off values are presented in relative units/ml.

### The two ELISA systems using the human cell-expressed ectodomains of Dsg 3 and Dsg 1 are useful for monitoring serum autoantibody levels of individual patients during the course of the disease

From 15 patients, sera were obtained at at least three different time points during the disease course. For all monitored individuals, ELISA values fluctuated in parallel with the clinical severity. Gradually decreasing levels of both anti-Dsg 3 and anti-Dsg 1 autoantibodies were accompanied by a decrease of disease activity scores while in patients with relapses, an increase of IgG reactivity paralleled rising severity scores. Disease activity scores and ELISA values were statistically analysed. A significant correlation was found between anti-Dsg 3 ELISA values and clinical scores in patients with PV ($P < 0.001$; Fig. 2a) and between anti-Dsg 1 ELISA values and clinical scores in PV ($P < 0.001$; Fig. 2b) and PF ($P < 0.001$; Fig. 2c).

## Discussion

Sensitive and specific detection systems for serum antibodies are important tools for diagnosis of pemphigus. Indirect IF microscopy on monkey oesophagus is not reactive with all pemphigus sera and does not differentiate between autoantibodies against Dsg 3 and 1, the main target antigens in PV and PF, respectively. To solve this problem, several ELISA systems have previously been developed (7,12–15). Two of them were based on baculovirus-encoded recombinant ectodomains of Dsg 3 and 1 and have been widely used after commercialization (16,17,19–26).

In the present study, the ectodomains of Dsg 3 and 1 were expressed in a human cell line to ensure post-

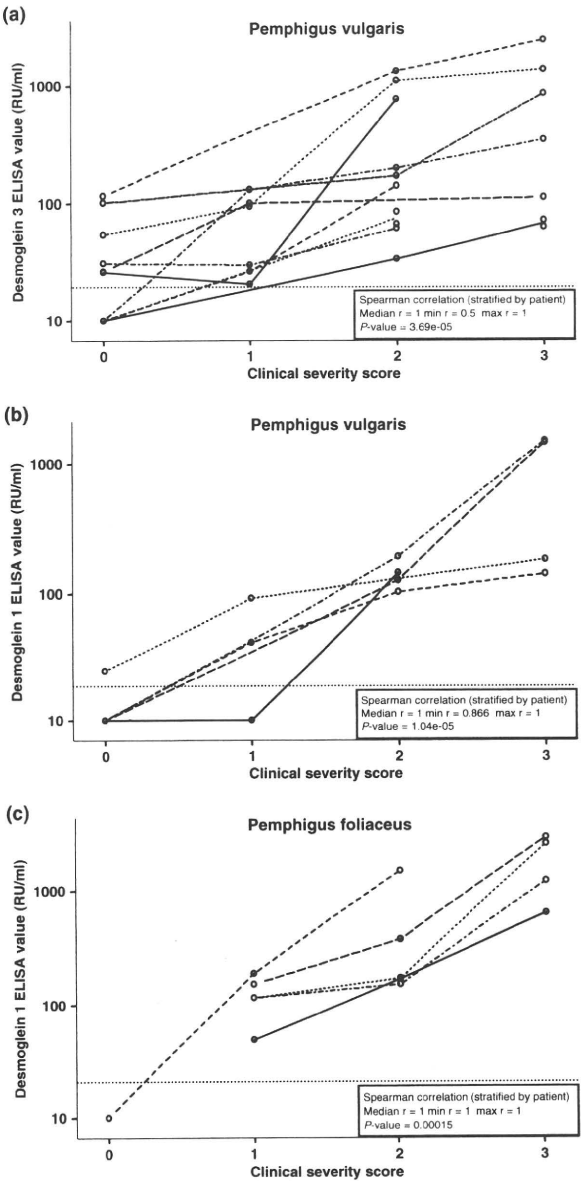


**Figure 2.** Statistical correlation of disease activities and serum levels of autoantibodies detected by the novel anti-Dsg 3 ELISA (a) and Anti-Dsg 1 ELISA (b,c) in patients with pemphigus vulgaris (PV; a,b) and pemphigus foliaceus (PF; c) during the course of the disease. Disease activity was scored in 10 Caucasian patients with PV and 5 with PF. More than 10 lesions corresponded to an activity score of 3, 4–10 lesions to a score of 2, 1–3 lesions to a score of 1, when all lesions had healed the score was 0. Sera were obtained at at least three different time points. Spearman correlation coefficients of disease score versus log-transformed ELISA values were calculated individually for each patient. By the use of the non-parametric Spearman test for independency with stratification for individual patients, a statistically significant correlation between clinical severity and autoantibody levels was demonstrated, indicating that the novel assays are suitable to monitor autoantibody levels during the course of the disease. RU, relative units.

translational protein processing with the utmost resemblance to the human epidermal proteins. Although the relevance of conformational and linear epitopes on Dsg 3 and 1 in pemphigus has not yet been fully elucidated (4–11) it is assumed that diagnostic tests using antigens with conformational epitopes as authentic as possible may be advantageous.

The novel ELISA systems were shown to be highly sensitive and specific for the detection of circulating anti-Dsg 3 antibodies in PV and anti-Dsg 1 antibodies in PF and may therefore be applied in the standard diagnosis of pemphigus. The sensitivities of the novel assays (anti-Dsg 3, 100%; anti-Dsg 1, 96%) were similar to the published sensitivities of the respective already commercially available MBL systems [anti-Dsg 3, 93.8% (12), 97.5% (23), 94.5% (24); anti-Dsg 1, 95.6% (12), 97.9% (23)]. Since reactivity with the MBL assays was part of the diagnostic criteria, direct comparison of sensitivities between the two systems in the present study was not possible. Specificities of the novel anti-Dsg 3/1 ELISA systems were slightly higher for the tested BP sera (97.9%/97.9%) compared to the conventional systems (95.8%/95.8%). While for healthy controls a specificity of 99.8% and 99.3% was achieved for the current anti-Dsg 3 and anti-Dsg 1 assays, a similar performance was obtained in the conventional systems by using a 'grey zone' between cut-off levels of 14 and 20 U/ml and 7 and 20 U/ml, respectively (12,23,25).

The pathogenic relevance of pemphigus autoantibodies has clearly been demonstrated (27,28). While antibodies against other keratinocyte cell surface proteins, including cholinergic receptors and annexins, have also been implicated into the pathogenesis of this disease (2), the pathogenic potential of pemphigus autoantibodies could only be fully depleted by preadsorption with recombinant Dsg 3 and 1, respectively (29,30). Accordingly, it has been repeatedly suggested that serum levels of anti-Dsg 3 and anti-Dsg 1 antibodies parallel disease activity during the course of PF and PV, respectively (7,12,17,20,21,31–33). Four of these studies provided a statistical analysis demonstrating a relationship between mean or median levels of anti-Dsg antibody levels and disease severity (7,20,21,32). However, no study has yet statistically analysed serial serum samples from individual patients taken at different time points during the disease course. Recently, the mature Dsg 1 and Dsg 3 isoforms were shown to preferentially recognise pathogenic patient autoantibodies compared to the proprotein isoform (34,35). HEK293 cells were shown to only express the mature Dsg isoforms which is most likely due to the high level of a potent furin-like propeptidase in these cells (36).

To exemplify the use of the novel ELISA systems beside their application as diagnostic tools a statistical correlation between clinical severity and serum levels of anti-Dsg 3 and anti-Dsg 1 antibodies in individual patients during the


 

course of their disease was performed. Our data show that serum anti-Dsg antibody levels reflect clinical activity during the disease course and may and indicate that monitoring of serum autoantibodies in pemphigus may be helpful. Whether autoantibody levels precede a relapse and can be used for guiding treatment decisions needs to be addressed in a prospective study.

In the present study, two sensitive and specific ELISA systems were established for the analysis of serum autoantibodies in pemphigus. The expression of the Dsg 3 and 1 ectodomains in the human cell line HEK293 ensured the exclusive expression of the mature Dsg isoforms and a most authentic availability of conformational epitopes. The novel assays revealed a statistically significant relation between clinical severity and autoantibody levels in individual patients with PV and PF. This observation indicates that the novel detection systems will also be helpful in monitoring disease activity in pemphigus.

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## References


1 Stanley J R, Amagai M. Pemphigus, bullous impetigo, and the staphylococcal scalded-skin syndrome. N Engl J Med 2006: **355**: 1800–1810.
2 Grando S A. Cholinergic control of epidermal cohesion. Exp Dermatol 2006: **15**: 265–282.
3 Beutner E H, Jordon R E. Demonstration of skin antibodies in sera of pemphigus vulgaris patients by indirect immunofluorescent staining. Proc Soc Exp Biol Med 1964: **117**: 505–510.
4 Amagai M, Ishii K, Hashimoto T, Gamou S, Shimizu N, Nishikawa T. Conformational epitopes of pemphigus antigens (Dsg1 and Dsg3) are calcium dependent and glycosylation independent. J Invest Dermatol 1995: **105**: 243–247.
5 Tsunoda K, Ota T, Aoki M *et al.* Induction of pemphigus phenotype by a mouse monoclonal antibody against the amino-terminal adhesive interface of desmoglein 3. J Immunol 2003: **170**: 2170–2170
6 Payne A S, Ishii K, Kacir S *et al.* Genetic and functional characterization of human pemphigus vulgaris monoclonal autoantibodies isolated by phage display. J Clin Invest 2005: **115**: 888–899.
7 Muller R, Svoboda V, Wenzel E *et al.* IgG reactivity against non-conformational NH-terminal epitopes of the desmoglein 3 ectodomain relates to clinical activity and phenotype of pemphigus vulgaris. Exp Dermatol 2006: **15**: 606–614.
8 Hacker M K, Janson M, Fairley J A, Lin M S. Isotypes and antigenic profiles of pemphigus foliaceus and pemphigus vulgaris autoantibodies. Clin Immunol 2002: **105**: 64–74.
9 Futei Y, Amagai M, Sekiguchi M, Nishifuji K, Fujii Y, Nishikawa T. Use of domain-swapped molecules for conformational epitope mapping of desmoglein 3 in pemphigus vulgaris. J Invest Dermatol 2000: **115**: 829–834.
10 Muller R, Svoboda V, Wenzel E, Muller H H, Hertl M. IgG against extracellular subdomains of desmoglein 3 relates to clinical phenotype of pemphigus vulgaris. Exp Dermatol 2008: **17**: 35–43.
11 Yeh S W, Cavacini L A, Bhol K C *et al.* Pathogenic human monoclonal antibody against desmoglein 3. Clin Immunol 2006: **120**: 68–75.
12 Ishii K, Amagai M, Hall R P *et al.* Characterization of autoantibodies in pemphigus using antigen-specific enzyme-linked immunosorbent assays with baculovirus-expressed recombinant desmogleins. J Immunol 1997: **159**: 2010–2017
13 Bhol K, Tyagi S, Natarajan K, Nagarwalla N, Ahmed A R. Use of recombinant pemphigus vulgaris antigen in development of ELISA and IB assays to detect pemphigus vulgaris autoantibodies. J Eur Acad Dermatol Venereol 1998: **10**: 28–35.
14 Breu-Velez A M, Yepes M M, Patino P J, Bollag W B, Montoya F Sr. A sensitive and restricted enzyme-linked immunosorbent assay for detecting a heterogeneous antibody population in serum from people suffering from a new variant of endemic pemphigus. Arch Dermatol Res 2004: **295**: 434–441.
15 Ide A, Hashimoto T, Amagai M, Tanaka M, Nishikawa T. Detection of autoantibodies against bullous pemphigoid and pemphigus antigens by an enzyme-linked immunosorbent assay using the bacterial recombinant proteins. Exp Dermatol 1995: **4**: 112–116.
16 Ng P P, Thng S T, Mohamed K, Tan S H. Comparison of desmoglein ELISA and indirect immunofluorescence using two substrates (monkey oesophagus and normal human skin) in the diagnosis of pemphigus. Australas J Dermatol 2005: **46**: 239–241.
17 Lenz P, Amagai M, Volc-Platzer B, Stingl G, Kirnbauer R. Desmoglein 3-ELISA: a pemphigus vulgaris-specific diagnostic tool. Arch Dermatol 1999: **135**: 143–148.
18 Rosenbach M, Murrell D F, Bystryn J C *et al.* Reliability and convergent validity of two outcome instruments for pemphigus. J Invest Dermatol 2009: **129**: 2404–2410.
19 Amagai M, Tsunoda K, Zillikens D, Nagai T, Nishikawa T. The clinical phenotype of pemphigus is defined by the anti-desmoglein autoantibody profile. J Am Acad Dermatol 1999: **40** (2 Pt 1): 167–170.
20 Sharma V K, Prasad H R, Khandpur S, Kumar A. Evaluation of desmoglein enzyme-linked immunosorbent assay (ELISA) in Indian patients with pemphigus vulgaris. Int J Dermatol 2006: **45**: 518–522.
21 Harman K E, Seed P T, Gratian M J, Bhogal B S, Challacombe S J, Black M M. The severity of cutaneous and oral pemphigus is related to desmoglein 1 and 3 antibody levels. Br J Dermatol 2001: **144**: 775–780.
22 Harman K E, Gratian M J, Seed P T, Bhogal B S, Challacombe S J, Black M M. Diagnosis of pemphigus by ELISA: a critical evaluation of two ELISAs for the detection of antibodies to the major pemphigus antigens, desmoglein 1 and 3. Clin Exp Dermatol 2000: **25**: 236–240.
23 Amagai M, Komai A, Hashimoto T *et al.* Usefulness of enzyme-linked immunosorbent assay using recombinant desmogleins 1 and 3 for serodiagnosis of pemphigus. Br J Dermatol 1999: **140**: 351–357.
24 Daneshpazhooh M, Chams-Davatchi C, Khamesipour A *et al.* Desmoglein 1 and 3 enzyme-linked immunosorbent assay in Iranian patients with pemphigus vulgaris: correlation with phenotype, severity, and disease activity. J Eur Acad Dermatol Venereol 2007: **21**: 1319–1324.
25 Kulkollakarn S, Wattanakrai P, Vachiramon V, Chalidapongse P. Evaluation of sensitivity and specificity of enzyme-linked immunosorbent assay (ELISA) for detecting antidesmoglein 1 and 3 in Thai patients with pemphigus vulgaris and foliaceus. J Med Assoc Thai 2008: **91**: 1663–1668.
26 Zagorodniuk I, Weltfriend S, Shtruminger L *et al.* A comparison of anti-desmoglein antibodies and indirect immunofluorescence in the serodiagnosis of pemphigus vulgaris. Int J Dermatol 2005: **44**: 541–544.
27 Schiltz J R, Michel B. Production of epidermal acantholysis in normal human skin in vitro by the IgG fraction from pemphigus serum. J Invest Dermatol 1976: **67**: 254–260.
28 Anhalt G J, Labib R S, Voorhees J J, Beals T F, Diaz L A. Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. N Engl J Med 1982: **306**: 1189–1196
29 Amagai M, Hashimoto T, Shimizu N, Nishikawa T. Absorption of pathogenic autoantibodies by the extracellular domain of pemphigus vulgaris antigen (Dsg3) produced by baculovirus. J Clin Invest 1994: **94**: 59–67.
30 Amagai M, Hashimoto T, Green K J, Shimizu N, Nishikawa T. Antigen-specific immunoadsorption of pathogenic autoantibodies in pemphigus foliaceus. J Invest Dermatol 1995: **104**: 895–901.
31 Aoyama Y, Tsujimura Y, Funabashi M, Sato M, Kamiya H, Kitajima Y. An experience for ELISA for desmoglein 1, suggesting a possible diagnostic help to determine the initial therapy for pemphigus foliaceus. Eur J Dermatol 2000: **10**: 18–21.
32 Abasq C, Mouquet H, Gilbert D *et al.* ELISA testing of anti-desmoglein 1 and 3 antibodies in the management of pemphigus. Arch Dermatol 2009: **145**: 529–535.
33 Cheng S W, Kobayashi M, Kinoshita-Kuroda K, Tanikawa A, Amagai M, Nishikawa T. Monitoring disease activity in pemphigus with enzyme-linked immunosorbent assay using recombinant desmogleins 1 and 3. Br J Dermatol 2002: **147**: 261–265.
34 Sharma P M, Choi E J, Kuroda K, Hachiya T, Ishii K, Payne A S. Pathogenic anti-desmoglein MAbs show variable ELISA activity because of preferential binding of mature versus proprotein isoforms of desmoglein 3. J Invest Dermatol 2009: **129**: 2309–2312.
35 Yokouchi M, Saleh M A, Kuroda K *et al.* Pathogenic epitopes of autoantibodies in pemphigus reside in the amino-terminal adhesive region of desmogleins which are unmasked by proteolytic processing of prosequence. J Invest Dermatol 2009: **129**: 2156–2166.
36 Varshavsky A, Kessler O, Abramovitch S *et al.* Semaphorin-3B is an angiogenesis inhibitor that is inactivated by furin-like pro-protein convertases. Cancer Res 2008: **17**: 6922–6931.