Diagnosis and models of epidermolysis bullosa acquisita

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Artem Vorobyev

from Rybinsk, Russian Federation

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First referee: Prof. Dr. med. Ralf Ludwig

Second referee: Prof. Dr. med. Peter König

Third referee: Prof. Dr. med. G. Hofbauer

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# Diagnosis and models of epidermolysis bullosa aquisita

Introduction

Autoimmune blistering diseases (AIBD) are characterized by the presence of circulating and tissue-bound antibodies to structural components of the skin and mucous membranes. Clinical symptoms of these diseases are dependent on the targeted antigen. All AIBD according to the affected antigen can be divided into 2 major groups: pemphigus and pemphigoid diseases (Table 1).

Disease group	Disease	Antigen
Pemphigus diseases	Pemphigus vulgaris	desmoglein 1 desmoglein 3
	Pemphigus foliaceus	desmoglein 1
	Paraneoplastic pemphigus	envoplakin, periplakin, desmoglein 1, desmoglein 3, desmoplakin I/II α2 makroglobulin-like 1, desmocollin
	IgA-pemphigus	dsg1, dsg3, desmocollin 1
Pemphigoid diseases	Bullous pemphigoid	BP180, BP230
	Pemphigoid gestationis	BP180
	Mucous membrane pemphigoid	BP180, BP230, laminin 332, α6β4-Integrin, collagen type VII
	Linear IgA-dermatosis	LAD-1, BP180 NC16A, collagen type VII
	Anti-p200 pemphigoid	laminin γ1, p200-antigen
	Epidermolysis bullosa acquisita	collagen type VII

|	Dermatitis herpetiformis Duhring	epidermal transglutaminase (TG3) tissue transglutaminase (TG2)

Table 1. Classification of autoimmune bullous diseases

Pemphigus diseases are characterized by the presence of antibodies against desmosomal proteins and intraepidermal blisters in histological examination (Figure 1 A, B, C). Pemphigoid diseases are caused by the antibodies, recognizing components of hemidesmosomes. Histologically subepidermal blisters are observed (Figure 1 D, E, F).
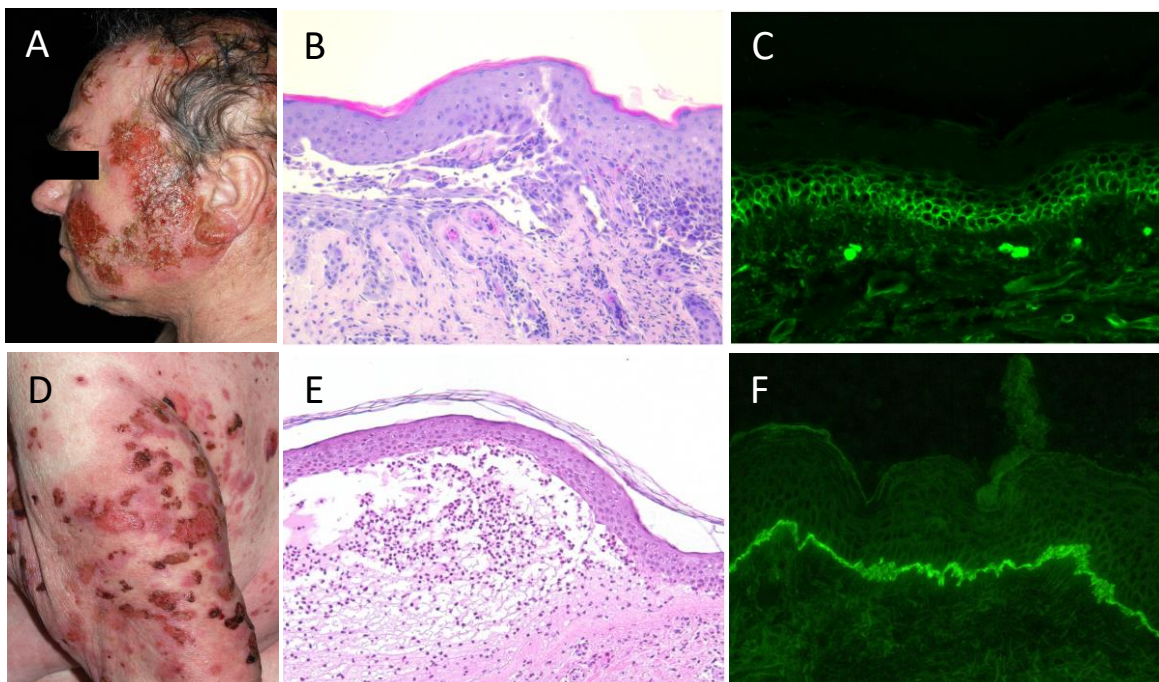
Figure 1. Typical clinical picture of pemphigus vulgaris shows multiple erosions (A). Histologically intraepidermal blisters are observed (B), in DIF typical intercellular depositions of IgG and complement (C). Clinical picture of bullous pemphigoid wih blisters, erosions, urticarial plaques (D). Microscopically subepidermal blisters (E) and linear deposions of IgG and complement C3 (F) are typical signs of pemphigoid diseases.

The "gold standard" of the diagnostic of autoimmune bullous diseases is detection of autoantibody-depositions in the epidermis (pemphigus diseases) or at the dermal-epidermal junction zone (pemphigoid diseases) in direct immunofluorescence. In pemphigoid diseases for the further differentiation of autoantibody specificity, indirect immunofluorescence on 1M NaCl-split skin can be used. After incubation of normal skin in 1M NaCl-solution, the split in the lamina lucida occurs, allowing detection of distinct antigens. At the roof of artificial blister BP180 and BP230 are detected, at the floor - col7, p200 and laminin 332 (Figure 2).
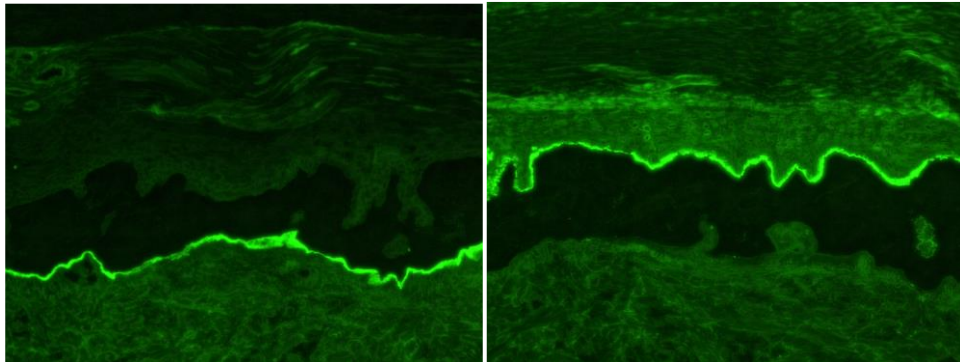
Figure 2. Indirect immunofluorescence on 1M salt-split skin. On the left - binding of autoantibodies to the floor of the artificial blister (col7, p200, laminin 332), on the right - to the roof of the blister (BP180, BP230)

Further specification of antigens is possible using serological assays such as enzyme-linked immunosorbent assay (ELISA) or western blot. For many frequently detected antigens, such as BP180 and BP230 ELISA kits are commercially available and assays can be performed in most of the laboratories in several hours. For rare diseases, such as EBA, anti-p200 pemphigoid or anti-laminin 332 pemphigoid no commercial kits have been available. Serologic diagnosis of epidermolysis bullosa acquisita is made by western blot with extract of human dermis, where 290 kDa (whole molecule of collagen type VII) and 145 kDa (NC1-domain) proteins are detected. Alternatively, ELISA with

immunodominant NC1 region of type VII collagen was previously tested, but is not widely available. Because western blot analysis is technically demanding and time consuming, it is available only in few diagnostic laboratories in the world, complicating diagnosis of epidermolysis bullosa acquisita.

During my work in the autoimmune diagnostics laboratory at the department of dermatology, University clinic Schleswig-Holstein I was involved in the analysis of the samples of a patient with clinical symptoms of epidermolysis bulosa dystrophica, such as nail loss and acral contractures (Figure 3).

Figure 3. Nail loss and acral contractures in a patient with EBA

Histopathologically, subepidermal blisters were detected, in the direct immunoflurescence linear depositions of IgG and complement C3 along the dermal-epidermal junction zone, suggesting the diagnosis of autoimmune subepidermal blistering disease. In further analysis of the autoantibody-specificity the targeted protein was recognized as type VII collagen (col7), antigen in epidermolysis bullosa acquisita (EBA). EBA is a rare chronic autoimmune subepidermal blistering disease of skin and mucous membranes. Its' incidence is estimated as 0,25-0,5 per 1 million/year. EBA is characterized by the presence of antibodies against col7. Col7 is a major component of anchoring fibrils,

connecting epidermis to dermis. Different clinical phenotypes of EBA are described, such as classic mechano-bullous and inflammatory variants. However, no pathogenic differences determining different clinical phenotypes have been described, most likely due to the low incidence of disease.

After that work I got interested in the pathogenesis of epidermolysis bullosa acquisita and its' different clinical phenotypes. For the further investigations, we collected to the moment biggest international cohort of epidermolysis bullosa acquisita patients (73 patients from Germany, Netherlands, Japan, South Korea, Great Britain).

This collection made the development of diagnostic tools possible (see 1 and 2). In addition, this cohort was the basis for fine epitope-mapping study (see 3).

Study 1.

Publication:

Komorowski L, Müller R, Vorobyev A, Probst C, Recke A, Jonkman MF, Hashimoto T, Kim SC, Groves R, Ludwig RJ, Zillikens D, Stöcker W, Schmidt E. Sensitive and specific assays for routine serological diagnosis of epidermolysis bullosa acquisita. J Am Acad Dermatol. 2013 Mar;68(3):e89-95 (IF: 5,004)

In collaboration with our partners from Euroimmun, Lübeck, we used our cohort of previously collected 73 EBA patients' sera to establish two sensitive and specific assays for detection of circulating anti-col7 antibodies. The diagnosis of epidermolysis bullosa acquisita was confirmed using indirect immunofluorescence of 1m salt-split skin and western blot with human dermal extract.

In detail, NC1 domain of type VII collagen was previously described as major antigenic epitope in epidermolysis bullosa acquisita. However, several cases of autoantibody reactivity to non-collagenous NC2 as well as to collagenous domain were reported. Because of low number of autoantibody reactivity outside NC1, we decided to use only NC1 domain in this assay.

Full-lengh non-collagenous NC1 domain, major antigen site of type VII collagen was cloned and expressed in HEK293 cells. The protein was coated on ELISA plates and tested with the sera of 73 EBA patients, to the moment largest reported EBA cohort. 395 control sera, including BP, anti-p200 pemphigoid, anti-laminin 332 pemphigoid and pemphigus vulgaris sera, were used.

Alternatively to ELISA, NC1-transfected HEK293 cells were coated on glass and used for indirect immunofluorescence microscopy. Treatment with secondary antibody allows the visualization of anti-collagen VII antibody bound to NC1-expressing HEK293 cells.

After optimisation of the ELISA cut-off, sensitivity and specificity of 98,7% were reached. In the indirect immunofluorescence sensitivity of 91,8% and specificity of 99,8% were observed.

Conclusion:

In this study we could develop two novel serological assays from diagnosis of epidermolysis bullosa acquisita, both demonstrating high sensitivity and specificity.

Study 2.

Publication:

Kim JH, Kim YH, Kim S, Noh EB, Kim SE, <u>Vorobyev A</u>, Schmidt E, Zillikens D, Kim SC. Serum levels of anti-type VII collagen antibodies detected by enzyme-linked immunosorbent assay in patients with epidermolysis bullosa acquisita are correlated with the severity of skin lesions. J Eur Acad Dermatol Venereol. 2013 Feb;27(2):e224-30 (IF: 3,105)

Serological assays are indispensable not only for the diagnostics of autoimmune bullous skin diseases, but also for the follow-up and treatment control. Previously, it has been reported that ELISA values are correlating with disease severity in bullous pemphigoid and pemphigus vulgaris, however, no studies on this matter in EBA have been performed. To address this question, we tested sera of 30 EBA patients in an ELISA with NC1 and NC2 domains (MBL, Nagoya, Japan) as well as in indirect immunofluorescence microscopy assay using NC1-domain expressing HEK293 cells (Euroimmmun, Lübeck, Germany). In this study we could confirm the previously reported sensitivity of 91,8 % and specificity of 98,1 % of the ELISA system. Indirect immunofluorescence on HEK293 cells showed comparable results. In our cohort of 30 EBA patients, ELISA values as well as IIF titres positively correlated to EBA severity. However, comparing ELISA and IIF titres before treatment and after remission, ELISA demonstrated better correlation with disease severity, while in IIF in one patient autoantibody titre increased after remission. In time course experiment, using the samples from one EBA patient collected at 7 different time points, ELISA values better represented disease severity that IIF titre.

Conclusion:

In this study we could demonstrate that serological assays, such as ELISA or IIF on HEK 293 cells are useful for the evaluation of EBA severity and follow up controls, as both assays positively correlated with disease severity score.

Study 3.

Clinically, different EBA phenotypes are described: (i) classical mechanobullous, and (ii) inflammatory type, including bullous pemphigoid-, Brunsting-Perry pemphigoid-, linear IgA dermatosis-, and mucous membrane pemphigoid-like variants. Mechanobullous variant of EBA resembles clinical picture of hereditary epidermolysis bullosa and is characterized by blisters at the trauma-prone sites, scarring, and milia formation. Inflammatory type of epidermolysis bullosa acquisita demonstrates erythematous plaques and tense blisters, mimicking bullous pemphigoid.

The pathogenesis of epidermolysis bullosa is relatively well characterised. First, antibodies bind to col7 at the dermal-epidermal junction, forming immune complexes. These immune complexes trigger complement activation cascade, which, in turn, recruits leukocytes to dermal-epidermal junction. Activated neutrophils secrete proteases, produce reactive oxygen species (ROS) and cause damage to dermal-epidermal junction, leading to blister formation. However, although these events are relatively well characterized, pathogenic mechanisms causing different clinical phenotypes are still not understood. It has been previously reported that NC1-domain of col7 interacts with collagen I,

collagen IV and laminin 332, so the perturbations of these interactions by autoantibodies could be one of the possible explanations.

To address this question, we created overlapping recombinant proteins, covering whole NC1 domain.

These recombinant proteins were tested in immunoblot assay with 69 clinically characterized EBA patients' sera. Autoantibodies of EBA patients recognized clusters of epitopes throughout the NC1 domain. To test if targeted epitopes correlate with clinical phenotype, homogeneity distribution analysis and Pearson's correlation analysis were used. No difference in epitope-recognition pattern and clinical phenotype of the patients could be detected. However, interestingly, epitope-recognition was strongly dependent on gender and age of the patients. In our opinion, antibody reactivity to NC2- and collagenous domains of collagen VII can not explain these differences, as only few patients have been described with autoantibody reactivity outside NC1 domain. We think another factors, such as neutrophil ability to mount an inflammatory response, may play a major role.

To test the pathogenic relevance of the epitope-specific antibodies, polyclonal rabbit anti-human col7 antibodies were produced. For the analysis of specificity of raised anti-collagen VII antibodies, these antibodies were tested in western blot with the proteins used for immunization. Interestingly, for several epitopes, binding of antibodies outside of the relevant epitope has been observed (Figure 7). This phenomenon of intramolecular cross-reactivity could be explained by high homology of FNIII-like domains and could contribute to pathogenicity of specific antibodies.

To test pathogenic relevance of single antibodies, ex vivo dermal-epidermal separation assay was used. Cryosections of normal human skin were treated with rabbit anti-human anti-col7 antibodies, followed by incubation with normal human PMNs. In this assay all of the used antibodies caused dermal-epidermal separation, which was dependent on PMNs, as incubation of skin sections with antibody alone did not cause split formation. For in vivo validation, antibodies against two subdomains of NC1 domain of type VII collagen were affinity-purified and injected into mice carrying null mutations of mouse COL7 and the human COL7 transgene. Both of these antibodies bound to the dermal-epidermal junction and induced typical histological changes and clinical phenotype in mice, for the first time demonstrating pathogenicity of anti-human collagen VII antibodies in vivo.

Conclusion:

This study provides novel insights into pathogenesis and diagnostics of epidermolysis bullosa acquisita, which will improve our understanding, diagnosis and treatment of this rare autoimmune disease.

Summary

Autoimmune blistering diseases (AIBD) are characterized by the presence of tissue-bound and circulating antibodies directed to the structural components of skin and mucous membranes. AIBD can be divided into two groups. Pemphigus diseases are characterized by the presence of autoantibodies to the desmosomal proteins; in the pemphigoid diseases autoantibodies target the components of dermal-epidermal junction. One of the typical pemphigoid diseases is epidermolysis bullosa acquisita.

Epidermolysis bullosa acquisita is a rare chronic autoimmune blistering disease of skin and mucous membranes, characterized by the presence of antibodies to collagen VII (col7), major component of anchoring fibrils. Col7 is a heterotrimer, consisting of three identical alpha-chains, each consisting of N-terminal non-collagenous NC1 domain, collagenous domain and C-terminal non-collagenous NC2 domain. In the previous studies, NC1 domain was described as major antigenic region of col7. Binding of autoantibodies to col7 causes formation of immune complexes and activation of complement cascade, which, in turn, recruits neutrophils to dermal-epidermal junction. Neutrophils secrete proteases and release ROS, causing tissue damage and blister formation. Clinically, mechanobullous and inflammatory EBA phenotypes are described.

Even though the pathogenesis of epidermolysis bullosa is relatively well characterised, pathogenic mechanisms causing different clinical phenotypes are still not understood. One of the possible explanations could be the perturbations of interaction of col7 NC1 with collagen I, collagen IV and laminin 332 by binding of autoantibodies to distinct subdomains of NC1. To address this question we collected to this moment largest reported cohort of 73 EBA patients. We cloned

and expressed recombinant overlapping proteins, covering whole NC1 domain of col7. The proteins were used for western blot assays, where no correlation of clinical phenotype with epitope-recognition pattern was detected. Interestingly, strong correlation of epitope-recognition pattern with gender and age of the patients was observed.

For the further validation of pathogenicity of antibodies against distinct NC1 subdomains, polyclonal rabbit anti-human col7 antibodies were raised. To test the crossreactivity of these antibodies, they were investigated in western blot assay with all of the recombinant proteins. Interestingly, intramolecular cross-reactivity was detected, what could be explained by high homology of FNIII-like subdomains of col7. All of the raised rabbit anti-human col7 antibodies demonstrated pathogenicity ex vivo in dermal-epidermal separation cryosection assay. For the in vivo validation, two antibodies were affinity-purified and injected into col7-humanized mice, causing clinical and histological phenotypes, similar to human EBA. Thus, in vivo pathogenicity of antibodies to human col7 was reported for the first time.

Diagnostic of EBA is based on the detection of tissue-bound and/or circulating autoantibodies to col7. Due to low incidence of EBA, diagnosis of this disease remains an issue, as no commercial kits for its serological diagnostic were available. Detection of circulating anti-col7 autoantibodies was possible only with technically demanding western blot analyses of human dermal extract or recombinant NC1 domain of col7, available only in several diagnostic laboratories in the world. Using our cohort of EBA patients, we were able to develop two novel serological diagnostic tools, namely ELISA with recombinant NC1 domain and Biochip with NC1-transfected HEK 293 cells. In our further study we could

demonstrate, that these tools can also be used for the evaluation of disease severity and treatment control.

In our studies using to the moment largest cohort of EBA patients, we could develop two novel diagnostic tools and imrove our understanding of pathogenesis of epidermolysis bullosa acquisita.

Deutsche Zusammenfassung

Blasenbildende Autoimmundermatosen (AIBD) sind durch die Präsenz der gewebsgebundenen und zirkulierenden Antikörper gegen Strukturproteine der Haut und Schleimhäuten gekennzeichnet. AIBD stellen eine heterogene Gruppe von Erkrankungen dar, die in zwei Gruppen aufgeteilt werden können. Pemphiguserkrankungen werden durch Antikörper gegen desmosomale Proteine verursacht, was sich histopathologisch als intraepidermale Spaltbildung darstellt. Bei den Pemphigoiderkrankungen zeigt sich eine subepidermale Spaltbildung, und die Autoantikörper sind gegen Strukturproteine der dermo-epidermalen Junktionszone gerichtet.

Eine repräsentative Erkrankung aus der Gruppe der Pemphigoid-Erkrankungen stellt die Epidermolysis bullosa acquisita dar.

Epidermolysis bullosa acquisita is eine chronische blasenbildende Autoimmunerkrankung der Haut und den Schleimhäuten, die durch Autoantikörper gegen Kollagen VII verursacht ist. Kollagen VII ist ein Heterotrimer, der aus drei identischen Alpha-Ketten besteht. Jede Alpha-Kette ist aus einer N-terminalen nicht kollagenosen NC1 Domäne, einer kollagenosen Domäne und einer C-terminalen nicht kollagenosen NC2 Domäne zusammengesetzt. In den vorherigen Studien wurde die NC1-Domäne bereits als der wichtigste antigene Bereich des Kollagen VII Molekül beschrieben. Die Bindung der Autoantikörper an Kollagen VII verursacht die Bildung der Immunkomplexen und Aktivierung der Komplementkaskade. Danach werden die Neutrophile zur dermal-epidermalen Junktionszone rekrutiert. Anschließend findet die Produktion der Proteasen und die Freisetzung der reaktiven Sauerstoffspezies durch die Neutrophile statt, was zur Gewebeschädigung und Blasenbildung führt.

Klinisch wurde der klassische mechanobullöse Phänotyp und der entzündliche Phänotyp der Epidermolysis bullosa acquisita beschrieben. Obwohl die Pathogenese der Epidermolysis bullosa acquisita relativ gut charakterisiert ist, die pathogenetische Mechanismen, die zu verschiedenen klinischen Phänotypen der Erkrankung führen sind noch nicht bekannt. Eine der möglichen Erklärungen könnte die Störung der Interaktion von Kollagen VII mit Kollagen I, Kollagen IV und Laminin 332 durch Bindung der Autoantikörper zu spezifischen Subdomänen der NC1-Domäne sein. Um diese Frage zu beantworten wurde die bisher größte Kohorte aus 73 Epidermolysis bullosa acquisita Patienten gesammelt.

Die rekombinanten überlappenden Proteine, die die gesamte NC1-Domäne abdecken, wurden von uns kloniert und exprimiert. Die Proteine wurden in Western Blot Untersuchungen mit Patientenseren getestet, wo keine Korrelation zwischen dem klinischen Phänotyp und dem Muster der durch die Antikörper gebundenen Epitopen festgestellt werden konnte. Interessanterweise, signifikante Korrelation des Musters der Epitopen mit Alter und Geschlecht der Patienten wurde bemerkt.

Um die Pathogenität der Antikörper gegen die spezifischen Fragmente der NC1-Domäne zu validieren, wurden in Kaninchen die poliklonale anti-humane Kollagen VII-Antikörper erzeugt. Um die Kreuzreaktivität dieser Antikörper zu untersuchen, wurde ein Western Blot mit allen rekombinanten Proteinen durchgeführt. Interessanterweise, wurde eine intramolekulare Kreuzreaktivität festgestellt, die durch eine hohe Homologie zwischen FNIII-Subdomäne erklärt werden könnte. Jeder dieser Kaninchen anti-human Kollagen VII Antikörper zeigte eine dermal-epidermale Trennung ex vivo bei der Untersuchung mit den Kryoschnitten normaler humaner Haut. Für die in vivo Validierung, zwei dieser Antikörper wurden affinitätsaufgereinigt und in die Kollagen VII-humanisierte

Mäuse injiziert. Klinisch und histologisch war der Phänotyp der humanen Epidemolysis bullosa acquisita ähnlich. Dadurch wurde die Pathogenität der gegen humanen Kollagen VII gerichteten Antikörper zum ersten Mal beschrieben.

Diagnosis der Epidermolysis bullosa acquisita wird durch Nachweis gewebsgebundener und/oder zirkulierender Antikörper gegen Kollagen VII gestellt. Wegen der geringen Inzidenz der Epidermolysis bullosa acquisita ist die Diagnostik dieser Erkrankung immer noch eine Herausforderung. Zu der Zeit der Untersuchung standen noch keine kommerzielle Diagnostikmethoden zur Vefügung. Nachweis von zirkulierenden anti-Kollagen VII Antikörper war nur mit der technisch sehr anspruchsvollen Western Blot Untersuchung des Extraktes humaner Dermis oder rekombinantem Kollagen VII möglich. Diese sind nur in einzelnen Diagnostiklaboren weltweit möglich. Mit unserer Kohorte der Epidermolysis bullosa acquisita Patienten wurden zwei neuen serologischen Diagnostikverfahren (ELISA mit rekombinantem NC1 und Biochip mit NC1-transfizierten HEK 293 Zellen) etabliert. In unserer nächsten Studie konnten wir zeigen, dass diese Verfahren auch für die Einschätzung des Schwierigkeitsgrads der Erkrankung sowie Behandlungskontrolle geeignet sind.

In unseren Studien mit der größten Kohorte der Epidermolysis bullosa acquisita Patienten konnten wir zwei neuen Diagnostikmethoden entwickeln und unsere Kenntnisse der Pathogenese der Erkrankung verbessern.

Publications:

1. **Vorobyev A**, Ujiie H, Recke A, Buijsrogge JJ, Jonkman MF, Pas HH, Iwata H, Hashimoto T, Kim SC, Hoon Kim J, Groves R, Samavedam U, Gupta Y, Schmidt E, Zillikens D, Shimizu H, Ludwig RJ. *Autoantibodies to Multiple Epitopes on the Non-Collagenous-1 Domain of Type VII Collagen Induce Blisters.* J Invest Dermatol. 2015 Jun;135(6):1565-73

2. Iwata H, Pipi E, Möckel N, Sondermann P, **Vorobyev A**, van Beek N, Zillikens D, Ludwig RJ. *Recombinant soluble CD32 supresses disease progression in experimental Epidermolysis bullosa aquisista.* J Invest Dermatol. 2015 Mar;135(3):916-9.

3. Recke A, Trog LM, Pas HH, **Vorobyev A**, Abadpour A, Jonkman MF, van Zandbergen G, Kauderer C, Zillikens D, Vidarsson G, Ludwig RJ. *Recombinant Human IgA1 and IgA2 Autoantibodies to Type VII Collagen Induce Subepidermal Blistering Ex Vivo.* J Immunol. 2014 Aug 15;193(4):1600-8.

4. Iwata H, Bieber K, Tiburzy B, Chrobok N, Kalies K, Shimizu A, Leineweber S, Ishiko A, **Vorobyev A**, Zillikens D, Köhl J, Westermann J, Seeger K, Manz R, Ludwig RJ. *B cells, dendritic cells, and macrophages are required to induce an autoreactive CD4 helper T cell response in experimental epidermolysis bullosa acquisita.* J Immunol. 2013 Sep 15;191(6):2978-88.

5. Kim JH, Kim YH, Kim S, Noh EB, Kim SE, **Vorobyev A**, Schmidt E, Zillikens D, Kim SC. *Serum levels of anti-type VII collagen antibodies detected by enzyme-linked immunosorbent assay in patients with epidermolysis bullosa acquisita are correlated with the severity of skin lesions.* J Eur Acad Dermatol Venereol. 2013 Feb;27(2):e224-30.

6. Komorowski L, Müller R, **Vorobyev A**, Probst C, Recke A, Jonkman MF, Hashimoto T, Kim SC, Groves R, Ludwig RJ, Zillikens D, Stöcker W, Schmidt E. *Sensitive and specific assays for routine serological diagnosis of epidermolysis bullosa acquisita.*J Am Acad Dermatol. 2013 Mar;68(3):e89-95.

7. Meissner C, Hoefeld-Fegeler M, Vetter R, Bellutti M, **Vorobyev A**, Gollnick H, Leverkus M. *Severe acral contractures and nail loss in a patient with mechano-bullous Epidermolysis bullosa acquisita.* Eur J Dermatol. 2010 Jul-Aug;20(4):543-4.

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Curriculum vitae

Personal details

Name   Artem Vorobyev

Date of birth   14.04.1982

e-mail   artem.vorobyev@uksh.de

Nationality   Russian

Education and qualifications

12/2012-present	Physician, Department of Dermatology, University of Lübeck
04/2009-12/2012	PhD student, Department of Dermatology, University of Lübeck
10/2007-03/2008	Internship, Department of Dermatology, Hospital Saint-Louis, Paris, France
09/2005-08/2007	Specialisation in dermatology, Medical University of Yaroslavl, Russia
09/1999-08/2005	Studies of human medicine, Medical University of Yaroslavl, Russia

Severe acral contractures and nail loss in a patient with mechano-bullous Epidermolysis bullosa acquisita

Epidermolysis bullosa acquisita (EBA) is a chronic acquired autoimmune blistering disease with highly variable clinical manifestations [1]. The classic mechano-bullous non-inflammatory type presents with acral blisters and erosions. Inflammatory variants of EBA can resemble bullous or cicatricial pemphigoid, and severe mucosal involvement may be present, leading to severe complications, such as oesophageal stenosis [2]. Therapeutically, EBA represents a major challenge, requiring high dose long term immunosuppressive therapy, with modest long term success [3].

We describe a 50-year-old patient in whom a mechano-bullous form of EBA was diagnosed 12 years ago [4]. First symptoms were blister formation on mechanically stressed areas (figure 1A). Furthermore, aggravated blister formation of the hands was evident within hours after sun exposure. Histopathology of biopsies revealed subepidermal bullae and a dense inflammatory infiltrate in the upper dermis. Direct immunofluorescence (DIF) microscopy showed linear depositions of IgG and C3 at the basement membrane zone, and indirect IF microscopy showed binding of circulating IgG (figure 1F), but not IgA antibodies to the dermal side of the split (figures 1G, H). Immunoblot analysis with IgG4 isotype-specific secondary antibodies showed that the patient's serum specifically reacted with the recombinant NC-1 domain of type VII collagen. ELISA using recombinant BP230, BP180 NC16A domains, as well as ANA, ENA, or Abs against single or double stranded DNA, were negative. We diagnosed a predominantly mechano-bullous form of EBA. The patient refused consent for an oral corticosteroid therapy and did not present again for several years. He noted an aggravation of the symptoms upon intensive manual work. 9 years after diagnosis he noted progressive atrophic scarring of his hands (figure 1B). After the initial diagnosis 12 years before, therapy with intravenous immunoglobulins (1.2 g kg^{-1} in monthly intervals over a total of 18 months) was initiated. Subsequently, he was treated with mycophenolate mofetil (2 g daily p.o.) and colchicine (1 mg daily) [5] for several years. Later, recurrent oesophageal strictures required balloon dilation. 12 years after the initial diagnosis, the patient presented with severe contractures of the hands and feet (figures 1C-E). The palmar side of his hands demonstrated severe dermatogenic contractures and nail loss (figure 1E). For this we started systemic corticosteroid treatment in combination with azathioprine (1.5 mg/kg body weight). Based upon previous successful reports [6, 7], we initiated 4 cycles of a B-cell depleting therapy with Rituximab (375 mg/m^2 infusion once a week) and achieved remission of the inflammatory clinical symptoms over 4 months. Circulating autoantibody titers against the immuno-dominant (NC-1) domains of type VII collagen (titer of 1:1600 at presentation) were undetectable within 4-6 months, and the skin vulnerability was substantially improved.

A loss of function of type VII collagen due to mutations within the COL7A1 gene leads to different forms of epi-

Figure 1. Mechano-bullous form of Epidermolysis bullosa acquisita leads to severe acral contractures. **A)** Erythematous papules and plaques with crusting of the right hand at the time of diagnosis. **B)** Right hand with initial contractures of the hand, severe atrophy and scars of the dorsal side of the hands; 9 years after diagnosis. The contracture of the PIP joint was 30° on both sides and the range of motion in the MCP joint 20°-70° on both sides. **C)** Development of severe acral contractures 12 years after diagnosis. Lateral look at the wrist with confluent erythematosus plaques. **D)** Palmar aspect of both hands with severe contractures of the distal and interphalangeal joints. **E)** Total destruction of the nail organs at digit 2-5 of the right hand. **F-H)** Indirect IF microscopy on 1 M NaCl split human skin (original magnification × 200 for F-H) demonstrated binding of circulating IgG antibodies (F) to the dermal side of the split, whereas IgA reactivity (H) was absent in our patient. H) A positive control patient with known IgA EBA demonstrates binding of IgA autoantibodies to the dermal side of the split.

dermolysis bullosa [8]. We demonstrate that over the prolonged course presented here a predominantly mechano-bullous EBA can lead to severe contractures and mutilations that may resemble a dystrophic form of Epidermolysis bullosa. To the best of our knowledge, this is the first documented case of such major skin complications of EBA in the literature, arguing for an early aggressive immunosuppression for such forms of EBA. Although the patient was not continuously affected in his daily life by the extent of active disease, the mechano-bullous form of EBA can lead to serious acral complications. We will now aim to mobilize the patient once clinical activity of the disease is controlled by immunosuppressive and B-cell depleting therapy. Future studies will evaluate the effectiveness of immunosuppression, not only for acute inflammation [9], but also for prevention of mutilations. We speculate that immunosuppression and thus reduction of circulating and tissue-bound autoantibodies to collagen type VII may delay the scarring and contractions that we document in this report. ■

[1]Department of Dermatology and Venereology, Otto-von-Guericke-University Magdeburg, Germany
[2]Department of Dermatology, Venereology, and Allergology, Medical Faculty Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany
[3]Department of Dermatology, University of Lübeck
<martin.leverkus@medma.uni-heidelberg.de>

Carl MEISSNER[1]
Marc HOEFELD-FEGELER[1]
Robert VETTER[1]
Michael BELLUTTI[1]
Artem VOROBYEV[3]
Harald GOLLNICK[1]
Martin LEVERKUS[1,2]

1. Sitaru C. Experimental models of epidermolysis bullosa acquisita. *ExpDermatol* 2007; 16: 520-31.
2. Shipman AR, Agero AL, Cook I, *et al.* Epidermolysis bullosa acquisita requiring multiple oesophageal dilatations. *ClinExpDermatol* 2008; 33: 787-9.
3. Schmidt E, Brocker EB, Goebeler M. Rituximab in treatment-resistant autoimmune blistering skin disorders. *ClinRevAllergy Immunol* 2008; 34: 56-64.
4. Jappe U, Zillikens D, Bonnekoh B, Gollnick H. Epidermolysis bullosa acquisita with ultraviolet radiationsensitivity. *BrJ Dermatol* 2000; 142: 517-20.
5. Bibas R, Gaspar NK. Ramos-e-Silva. Colchicine for dermatologic diseases. *JDrugs Dermatol* 2005; 4: 196-204.
6. Niedermeier A, Eming R, Pfutze M, *et al.* Clinical response of severe mechanobullous epidermolysis bullosa acquisita to combined treatment with immunoadsorption and rituximab (anti-CD20 monoclonal antibodies). *Arch Dermatol* 2007; 143: 192-8.
7. Schmidt E, Benoit S, Brocker EB, Zillikens D, Goebeler M. Successful adjuvant treatment of recalcitrant epidermolysis bullosa acquisita with anti-CD20 antibody rituximab. *Arch Dermatol* 2006; 142: 147-50.
8. Dang N, Murrell DF. Mutation analysis and characterization of COL7A1 mutations in dystrophic epidermolysis bullosa. *Exp Dermatol* 2008; 17: 553-68.
9. Takae Y, Nishikawa T, Amagai M. Pemphigus mouse model as a tool to evaluate various immunosuppressive therapies. *Exp Dermatol* 2009; 18: 252-60.

Secondary syphilis presenting in a red tattoo

A 26-year-old Chinese male presented with a reddish brown scaly maculopapular eruption on a red tattoo on his trunk, and with slight pruritus for about 6 months. The initial lesions occurred on his palms. The red area of the tattoo was involved although the dark blue area of the tattoo was free of lesions. One year before, the patient had a dragon tattooed on his trunk with bulk dark ink and a few cinnabars, by a professional tattoo artist. He had a history of unprotected sexual activities during the previous 12 months. No history of genital ulcer or other cutaneous or systemic disease could be elicited from the patient. There was no history of hair loss.

Physical examination revealed reddish brown macules and papules with slight scales, which covered most of the red area of the tattoo. The dark blue area was not involved *(figure 1A)*. A scaly circular rash was present on his palms. There was no palpable lymphadenopathy. Mucous membranes of the mouth and pharynx were unremarkable. A biopsy from the lesion on the red tattoo revealed parakeratosis, acanthosis and numerous neutrophils present within the epidermis; a diffused infiltrate of plasma cells associated with red and black pigment-containing macrophages was present in the upper dermis *(figure 1B)*. Warthin-Starry staining was performed and was negative for spirocheta. Laboratory tests including blood, urine, stools, hepatic and renal functions were all within normal limits. A patch test for cinnabar was negative. Repeated fungal tests under microscopy and cultures were negative. The result of rapid plasma reagin (RPR) was positive with titre 1:32. *Treponema pallidum* hemagglutination assay (TPHA) exhibited a positive result with titre 1:2560. HIV antibody was negative. A diagnosis of secondary syphilis was made. Procaine benzylpenicillin was prescribed, 800,000 units per day for 2 weeks. Lesions of the trunk and palms cleared completely about 4 weeks later. The RPR titre decreased to 1:2.

Syphilis is a chronic sexually transmitted disease caused by *Treponema pallidum*. Its incidence has risen in the last few decades [1]. Up to now, the relationship between tattoos and syphilis is uncertain. It was believed that the needles used during tattooing and the saliva used
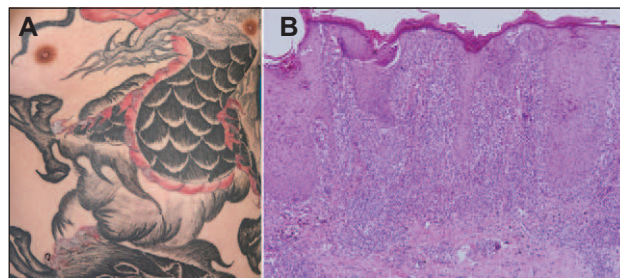
Figure 1. A) Macules and papules with slight scales covered most of the red tattoo. **B)** Diffused infiltrate of plasma cells associated with red and black pigment-containing macrophages present in the upper dermis (hematoxylin-eosin, original magnification ×100).

Sensitive and specific assays for routine serological diagnosis of epidermolysis bullosa acquisita

Lars Komorowski, PhD,[a] Ralf Müller, PhD,[b] Artem Vorobyev, MD,[b] Christian Probst, PhD,[a] Andreas Recke, MD,[b] Marcel F. Jonkman, MD, PhD,[c] Takashi Hashimoto, MD,[d] Soo-Chan Kim, MD,[e] Richard Groves, MD, FRCP,[f] Ralf J. Ludwig, MD,[b] Detlef Zillikens, MD,[b] Winfried Stöcker, MD,[a] and Enno Schmidt, MD, PhD[b,g]

Luebeck, Germany; Groningen, The Netherlands; Kurume, Japan; Seoul, Korea; and London, United Kingdom

Background: Epidermolysis bullosa acquisita (EBA) is a severe autoimmune subepidermal blistering disease characterized by autoantibodies against the N-terminal collagenous domain (NC1) of type VII collagen (Col VII).

Objective: Development of reliable assays for the detection of anti-Col VII-NC1 antibodies.

Methods: NC1 was expressed in human HEK293 cells and used as target antigen in an enzyme-linked immunosorbent assay (ELISA) and in an immunofluorescence assay (IFA). These two assays were probed in a large cohort of patients with EBA (n = 73), bullous pemphigoid (BP, n = 72), anti-p200 pemphigoid (n = 24), anti-laminin 332 mucous membrane pemphigoid (MMP, n = 15), pemphigus vulgaris (PV, n = 24), and healthy control subjects (n = 254).

Results: The cut-off for the ELISA was optimized for accuracy by receiver-operating characteristics (area under the curve [AUC] = 0.9952). IgG reactivity against NC1 was detected in 69 of 73 EBA (94.5%) and 5 control sera (2 healthy controls and 3 BP patients), resulting in a specificity of 98.7%. The IFA showed a sensitivity of 91.8% and specificity of 99.8%. Reproducibility of the ELISA was demonstrated by an intra-class correlation coefficient of 0.97. IgG subclass analyses by ELISA revealed IgG1, IgG2, IgG3, and IgG4 anti-NC1 reactivity in 83.6%, 85.3%, 37.7%, and 83.6% of EBA sera, respectively.

Limitations: The novel assays were not evaluated prospectively and their use in monitoring serum levels during the disease course was not tested.

Conclusion: The two assays are highly specific and sensitive to diagnose EBA. Their diagnostic competence was demonstrated in a large cohort of well-characterized EBA sera. (J Am Acad Dermatol 2013;68:e89-95.)

Key words: autoantibody; ELISA; immunofluorescence; type VII collagen.

INTRODUCTION

Epidermolysis bullosa acquisita (EBA) is a rare chronic subepidermal bullous autoimmune disease characterized by autoantibodies against type VII collagen (Col VII).[1,2] Clinically, mechanobullous (trauma-induced) and inflammatory variants (resembling bullous pemphigoid or mucous membrane pemphigoid) are differentiated.[3-5] Diagnosis is made by the presence of IgG and/ or IgA antibodies at the dermoepidermal junction by direct immunofluorescence (IF) microscopy.[6-8] Recently, a diagnostic "u-serrated" binding pattern at the dermoepidermal junction has been described that may differentiate EBA from other subepidermal blistering autoimmune diseases by direct IF microscopy.[9,10] Circulating autoantibodies in EBA patients bind to the floor of 1 mol/L NaCl-split normal human skin by indirect IF microscopy and recognize the 290-kd type VII collagen by Western blotting with human dermis.[1,2,11]

Col VII, the main constituent of anchoring fibrils, is a homotrimer of 3 identical α-chains. Each 290-kd chain is composed of a central collagenous triple helical rod flanked by an N-terminal 145-kd noncollagenous domain (NC1) and a 34-kd NC2 domain (Fig 1). A portion of the NC2 domain is removed when Col VII molecules form tail-to-tail dimers that are stabilized by disulfide bonding between the remaining NC2 domains.[12,13] The NC1 domain has previously been identified as the immunodominant region of Col VII.[14-18]

The clinical picture together with positive u-serrated binding pattern by direct IF microscopy are sufficient for the diagnosis.[9,10] However, serology is supportive when positive, and mandatory if the serration pattern is not recognized. At present, serological diagnosis is made by the detection of serum autoantibodies against Col VII by Western blotting with extract of human dermis, conditioned medium of human WISH cells, and the pepsinized human protein.[2,5,19] Alternatively, the immunodominant NC1 domain has previously been employed by ELISA to specifically detect circulating anti-Col VII antibodies in 24, 15, and 49 EBA patients, respectively.[18,20,21] To date, none of these test systems is widely available.

In our study, applying a large cohort of well-characterized EBA sera, we developed two highly

CAPSULE SUMMARY

- Patients with epidermolysis bullosa acquisita have autoantibodies against collagen VII.

- We have developed 2 novel assays to determine them.

- The assays can help in the clinical practice to establish the correct diagnosis and monitor therapy.

specific and sensitive assays for the detection of serum anti-Col VII autoantibodies. of which the IF microscopy test will be widely available.

METHODS
Human sera

Sera from patients with EBA (n = 73) were collected at the dermatology departments in Luebeck (Germany), Groningen (The Netherlands), Kurume (Japan), Seoul (South Korea), and London (UK). All EBA sera (1) were taken from patients with a compatible clinical picture in the active stage of the disease, (2) were labeled the dermal side of human salt-split skin by indirect IF microscopy, and (3) reacted either with a 290-kd protein by Western blotting with extract of human dermis[22] or failed to react against the p200 protein, laminin γ1, and laminin 332 by Western blotting with dermal extract, recombinant C-terminus of laminin γ1, or extracellular matrix of cultured human keratinocytes, respectively.[23-25]

As control sera from patients with bullous pemphigoid (BP, n = 72), anti-laminin 332 mucous membrane pemphigoid (MMP, n = 15), anti-p200/ laminin γ1 pemphigoid (n = 24), and pemphigus vulgaris (PV, n = 24) as well as from healthy blood donors (HBD, n = 254) were used. All sera were stored at -20°C until assayed. Patients and control subjects gave written consent to participate in this study, which was adherent to the Declaration of Helsinki Guidelines and which was approved by the local Ethics Committee (10-017).

Cloning and expression of the NC1 domain of human type VII collagen

Full-length cDNA of the NC1 domain of human Col VII alpha 1 (accession number NM_000094) was amplified using appropriate primers (MWG Biotech, Ebersberg, Germany). The cDNA was ligated with vector pTriEx-1 (Novagen, Darmstadt, Germany) to give the construct pTriEx-1-pre-Col7A1NC1-His. Alternatively, a linker DNA coding for the transmembrane domain of desmoglein 1 was introduced into pTriEx-1-NcoI/XhoI, resulting in the construct pTriEx-1-pre-Col7A1NC1-TM. Both constructs were verified by DNA sequencing (MWG Biotech). Expression and purification of the soluble human NC1 domain of Col VII

Abbreviations used:

BP: bullous pemphigoid
Col VII: type VII collagen
DEJ: dermoepidermal junction
EBA: epidermolysis bullosa acquisita
ELISA: enzyme-linked immunosorbent assay
IF: immunofluorescence
NC1: noncollagenous domain 1
PBS: phosphate-buffered saline

(ColVII-NC1) from culture supernatant was conducted as described for the production of recombinant desmoglein ectodomains.[26]

## ELISA using the recombinant NC1 domain of human type VII collagen

Microtiter plates (Greiner, Frickenhausen, Germany) were coated with recombinant ColVII-NC1; serum samples were diluted 1:101. Subsequently, horse radish peroxidase-conjugated detection antibodies (anti pan-IgG, anti-IgG1, anti-IgG2, anti-IgG3, anti-IgG4; The Binding Site, Schwetzingen, Germany) were applied and IgG binding was visualized with TMB substrate (EUROIMMUN). Optical density (OD) was measured at 450 nm (reference, 620 nm). Samples were run in duplicate and results were expressed as mean values. The cut-off for positivity was validated and optimized by receiver-operating characteristics (ROC). A highly positive index patient serum was used to generate a standard curve consisting of 3 calibrators (1:50, 1:100, and 1:200 dilution) covering the linear range of the assay (0-4 RU/mL). Relative units (RU) were calculated from the OD values by this standard curve.

Immunofluorescence microscopy using membrane-bound recombinant NC1 domain of human type VII collagen

Alternatively to the production of soluble NC1, HEK293 were transfected with pTriEx-1-pre-Col7A1NC1-TM while growing on cover glasses. After 48 hours, cells were fixed with 1% (wt/vol) formaldehyde, acetone, ethanol, or mixtures thereof. Coated cover glasses were cut into millimeter-sized fragments (biochips) and used side by side with untransfected cells as substrates by indirect IF microscopy (Fig 2, A). Slides were incubated with human sera diluted 1:10 according to the standard protocol for indirect IF microscopy (Euroimmun).

Statistical analysis

For statistical analyses Gnu R open access software was used (R Development Core Team 2009; R Foundation for Statistical Computing, Vienna,
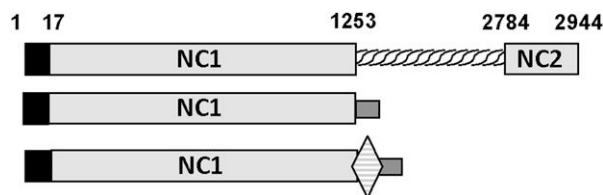

Fig 1. Schematic diagram of type VII collagen (Col VII, *top*) and the HEK293 cell-expressed recombinant non collagenous domain-1 (NC1) of Col VII used for ELISA (*middle*) and immunofluorescence microscopy (*bottom*). Col VII is a homotrimer consisting of an amino-terminal signal sequence (*black box*), the NC1 domain, a collagenous helical domain, and the NC2 domain. Amino acid numbers are indicated above. *Gray box*, 6×His tag; *diamond*, transmembrane domain of desmoglein 1.

Austria). The cutoff which maximized test accuracy was calculated by ROC analysis with package "DiagnosisMed" and inter-test reliability with package "psych".

RESULTS

The analysis of the 73 EBA and 395 control sera by ELISA revealed a high overall diagnostic performance, which is detailed in Table I. IgG reactivity to ColVII-NC1 was found in 69 of 73 (94.5%) EBA sera, 3 of the 72 BP sera (4.2%), 2 of 254 HBD sera (0.5%), and none of the PV, MMP, and anti-p200/laminin γ1 pemphigoid sera (Fig 3). To evaluate the reproducibility of the novel ELISA, the intraclass correlation coefficient (ICC) was calculated for intra-assay and interassay variation. The intra-assay variation determined by quadruplicate measurements on the same plate with sera (n = 55) covering a wide range of ELISA reactivities resulted in an ICC2 for randomly selected measurements of 0.975 (95% confidence interval: 0.961-0.984) (Fig 4, *upper panel*). The interassay variation, based on 8 different sera assayed in 6 separate experiments on different days, revealed an ICC3 for separate experiments of 0.973 (95% confidence interval 0.932-0.994), demonstrating a very good reproducibility for the novel assay (Fig 4, *lower panel*). IgG subclass reactivities were optimized using EBA sera with the highest remaining volume (n = 61) and randomly selected HBD sera (n = 151). IgG1, IgG2, IgG3, and IgG4 subclass autoantibodies against ColVII-NC1 were detected in 83.6%, 85.3%, 37.7%, and 83.6% of EBA sera, respectively.

As an alternative to ELISA, indirect IF microscopy using ColVII-NC1 expressed on the surface of HEK293 cells was used to analyze EBA and control sera (Fig 2, *B*). Fixation experiments revealed the best performance for formalin-only fixed cells due to the eradication of reactivities against intracellular
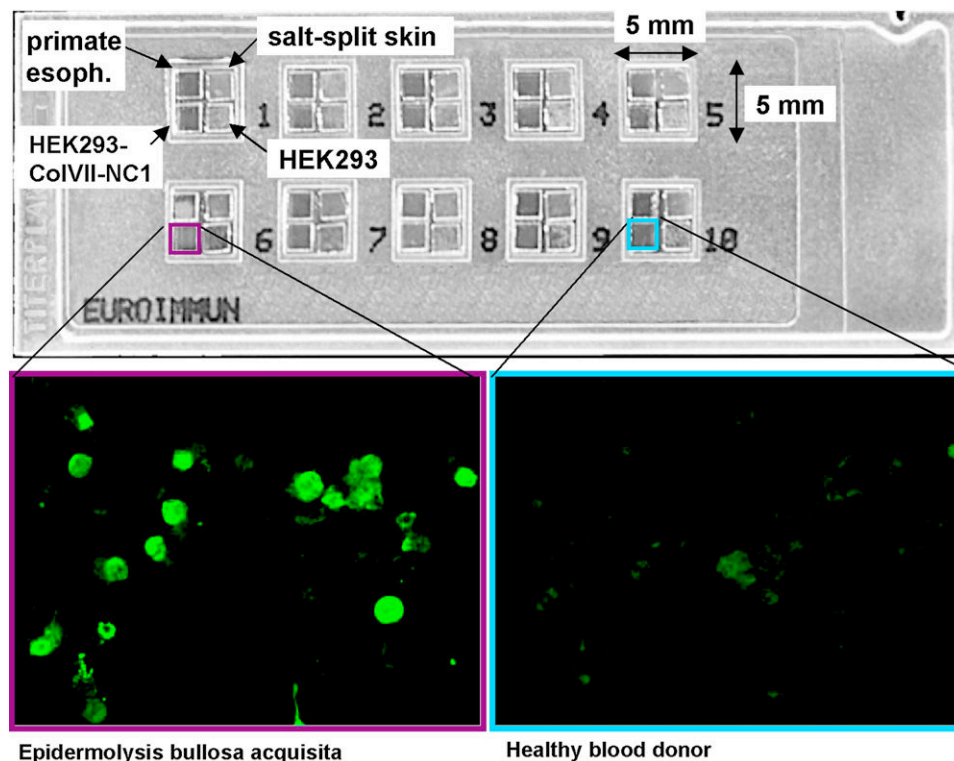
Fig 2. Slide with 10 reaction fields containing HEK293 cells expressing non-collagenous domain-1 of type VII collagen on cell surface via the transmembranous domain of desmoglein 1 (HEK293-ColVII-NC1) and untransfected control cells (HEK293). In the reaction field, additional substrates may be included containing other relevant target antigens or tissues (eg, primate esophagus [esoph.] and human salt-split skin. Autoantibodies in the serum of a patient with EBA labeled ColVII-NC1-expressing cells but not non-ColVII-NC1-expressing cells used as internal control (*lower left panel*). No reactivity of both ColVII-NC1-expressing and non-ColVII-NC1-expressing cells is seen with serum from a healthy blood donor (*lower right panel*).

components (that is, nuclear constituents targeted by common antinuclear antibodies), thereby easing evaluation of low-titer sera (data not shown). Storing experiments revealed that dust-free storing of desiccated slides containing ColVII-NC1−expressing and control cells at −20°C for up to 12 months did not affect the outcome of individual tests (data not shown).

Applying this novel IF microscopy test, 67 of 73 (91.8%) EBA sera, one of 72 (1.4%) BP sera, and none of the 154 HBD, 24 PV, 15 MMP, and 24 anti-p200/laminin γ1 pemphigoid sera were positive, resulting in a sensitivity of 91.8% (CI = 83.2%-96.2%) and a specificity of 99.8% (CI = 97.6%-100%). Full reproducibility was demonstrated on the basis of 3 subsequent slide lots and all criteria for a CE labeling could be fulfilled.

DISCUSSION

Patients with EBA need to be differentiated from patients with other subepidermal blistering disorders since EBA is usually more difficult to treat compared with, for example, BP and anti-p200/laminin γ1 pemphigoid[19] and may be associated with inflammatory bowel disease (reviewed in Hundorfean et al[27]). The commonly employed indirect IF microscopy using tissue substrates is not sufficient for the differentiation of EBA from pemphigoid diseases, but combining it with serration pattern analysis by direct IF microscopy, it might be conclusive.[10,28] Nevertheless, demonstration of binding of autoantibodies to ColVII is definite for the diagnosis. Therefore, a number of ColVII-specific assays, mostly immunoblot and ELISA protocols, have been developed.[2,5,14-18,20,21] In particular, Chen et al[18] have previously developed a highly sensitive and specific ELISA based on the NC1 domain of ColVII expressed in human HEK293 cells. They subsequently demonstrated a higher diagnostic sensitivity of this assay compared with indirect IF microscopy on salt-split skin and Western blotting with recombinant and cell-derived ColVII.[18]

Table I. characteristics of the novel ColVII-NC1 ELISA

| Parameters	ColVII-NC1	95% CI
AUC	0.9905	0.9905-0.9999
Sensitivity	0.9452	0.8674-0.9785
Specificity	0.9897	0.9738-0.9960
Cut off[*,†]	0.68	
Accuracy	0.981	0.963-0.99
Maximum sum of sensitivity and sensitivity	1.931	1.863-1.971

AUC, Area under the curve; *CI*, confidence interval.
*Cut-off values are presented in relative units per milliliter.
†The optimal cut-off was determined by optimization of maximum accuracy.

Available diagnostic studies, however, included only a limited number of EBA and control sera and reported test systems remained in the realm of specialized laboratories. The aim of the present study was to develop a simple, sensitive, specific, and widely available assay for the serological diagnosis of EBA. In a first step, we established the ELISA by Chen et al[18] using the HEK293 cell–expressed NC1 domain of ColVII as the diagnostic "gold standard". After optimization of the ELISA cut-off for maximum test accuracy by ROC, the largest cohort of EBA sera to date (n = 73), complemented by a substantial control group of relevant diseases (n = 135) and a large number of sera from healthy individuals (n = 254), were probed. Our ELISA showed a high sensitivity of 98.7% and specificity (98.7%) comparable with the ELISA by Chen et al[18] as well as others [20,29] When the same sera were applied in the novel biochip IF test, sensitivity and specificity of 91.8% and 99.8%, respectively, were observed. Importantly, none of the sera from patients with anti-laminin 332 MMP, anti-p200/laminin γ1 pemphigoid, and PV and only 2 of 72 BP sera contained anti-ColVII-NC1 reactivity. One of these 2 BP sera also recognized the 290-kd band by immunoblotting with dermal extract and showed both dermal and epidermal binding by indirect IF microscopy on human salt-split skin, suggesting a possible overlap of BP and EBA. This serum was excluded from analyses for specificity in both the ELISA and the IF microscopy test.

In order to employ the novel IF microscopy test in the routine diagnosis of EBA, it was also optimized with regard to cell fixation, reproducibility, and storability. It has recently been CE-labeled, with Food and Drug Administration approval in progress. Moreover, within the same reaction field, the miniature biochips containing ColVII-NC1–expressing and control HEK293 cells can be placed next to
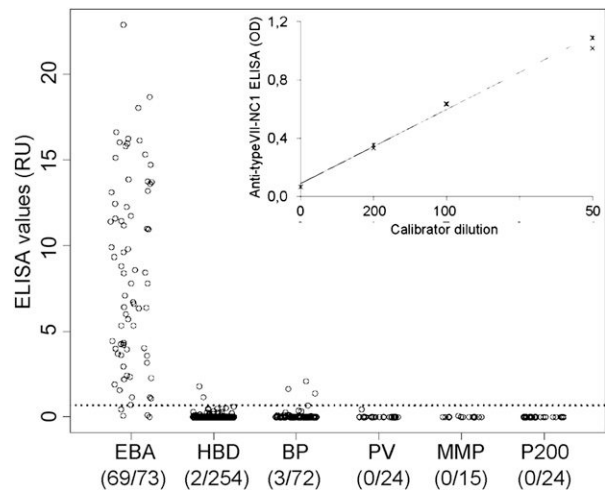
Fig 3. ELISA reactivities of sera from patients with EBA and controls. IgG reactivity to the noncollagenous domain-1 of type VII collagen was found in sera from 69 of 73 (94.5%) EBA patients, 2 of 254 (0.8%) healthy blood donors (HBD), 3 of 72 (4.1%) bullous pemphigoid (BP) patients and none of 24, 15, and 24 patients with pemphigus vulgaris (PV), anti-laminin 332 mucous membrane pemphigoid (MMP), and anti-p200/laminin γ1 pemphigoid, respectively. *Dashed line* indicates the cut-off value. A typical standard curve revealed a linear range of the ELISA (0-4 RU/mL; calibrators diluted 1:50, 1:100 and 1:200) and is shown as insert. RU was calculated from the OD values by this standard curve.

biochips containing other relevant target antigens or tissues, for example, primate esophagus and salt-split human skin, as shown in Fig 2, *A*. The biochip technique is currently developed to allow the simultaneous testing of up to 16 different substrates with a test volume of only 25 μl of 1:10 diluted serum.

In the present study, we focused on autoantibody reactivity against the NC1 domain of ColVII. In fact, only a few EBA patients have been described with autoantibody reactivity against the NC2 domain or the central rod.[22,30-33] Tanaka et al[30] and Ishii et al[33] reported on 1 and 5 patients, respectively, with exclusive reactivity against the central rod of Col VII. Furthermore, Tanaka et al[30] described 2 patients with NC2-specific autoantibodies, but no NC1 reactivity. These observations led to the combined use of the NC1 and NC2 domains in a recently developed ELISA. In the relatively large group of 49 EBA sera, only one serum exclusively reacted with the NC2 domain.[21] In the present study (data not shown) and earlier reports, all EBA sera recognized the NC1 domain.[14,15,18,20,29] We conclude that including the NC2 domain in the diagnostic assessment of EBA patients, particularly produced with a bacterial expression system, may only lead to a minimal increase in sensitivity while the specificity may be lower (eg,
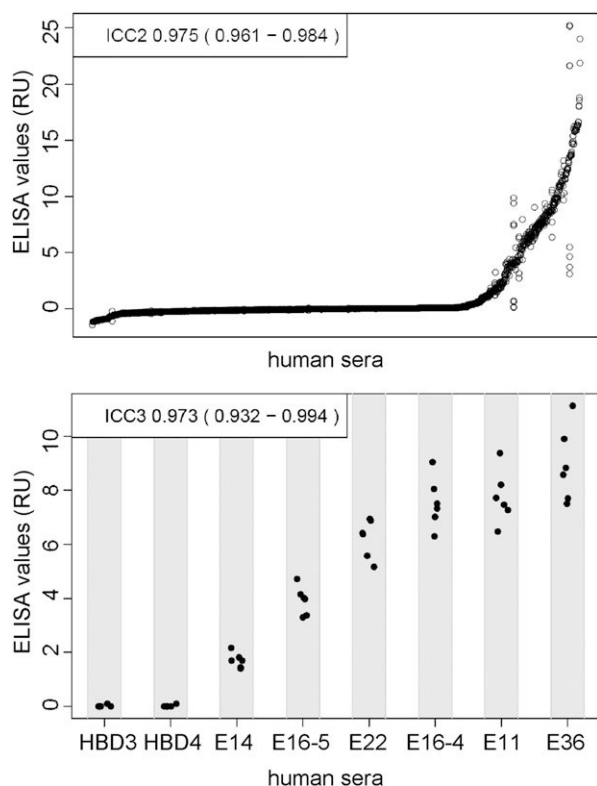
Fig 4. Reproducibility of ELISA measurements. The intra-assay variation was determined by quadruplicate measurements on the same plate with sera (n = 55) covering a broad range of ELISA reactivities. The intraclass coefficient for randomly selected measurements (ICC2) was 0.975 (95% confidence interval [CI]: 0.961-0.984). Each dot represents a single measurement. Results were sorted from left to right according to the median of quadruplicate measurements (*upper panel*). For determination of inter-assay variation, 8 different sera were measured in 6 different experiments on different days. The intraclass coefficient for jointly measured values (ICC3) was 0.973 (95% I: 0.932-0.994).

98.1% in the ELISA reported by Saleh et al[21] compared with 98.7% and 99.8% in the present study. To evaluate the potential of the novel assays to provide help for treatment decisions during the course of the disease, future studies are required that correlate serum anti-ColVII antibody levels with the disease activity during the follow-up of patients.

In summary, the two new assays are highly valuable for the serological diagnosis of EBA.

REFERENCES
1. Woodley DT, Briggaman RA, O'Keefe EJ, Inman AO, Queen LL, Gammon WR. Identification of the skin basement-membrane autoantigen in epidermolysis bullosa acquisita. N Engl J Med 1984;310:1007-13.
2. Woodley DT, Burgeson RE, Lunstrum G, Bruckner-Tuderman L, Reese MJ, Briggaman RA. Epidermolysis bullosa acquisita antigen is the globular carboxyl terminus of type VII procollagen. J Clin Invest 1988;81:683-7.
3. Roenigk HH Jr, Ryan JG, Bergfeld WF. Epidermolysis bullosa acquisita. Report of three cases and review of all published cases. Arch Dermatol 1971;103:1-10.
4. Caux F. Diagnosis and clinical features of epidermolysis bullosa acquisita. Dermatol Clin 2011;29:485-91.
5. Woodley DT, Remington J, Chen M. Autoimmunity to type VII collagen: epidermolysis bullosa acquisita. Clin Rev Allergy Immunol 2007;33:78-84.
6. Nieboer C, Boorsma DM, Woerdeman MJ, Kalsbeek GL. Epidermolysis bullosa acquisita. Immunofluorescence, electron microscopic and immunoelectron microscopic studies in four patients. Br J Dermatol 1980;102:383-92.
7. Yaoita H, Briggaman RA, Lawley TJ, Provost TT, Katz SI. Epidermolysis bullosa acquisita: ultrastructural and immunological studies. J Invest Dermatol 1981;76:288-92.
8. Zambruno G, Manca V, Kanitakis J, Cozzani E, Nicolas JF, Giannetti A. Linear IgA bullous dermatosis with autoantibodies to a 290 kd antigen of anchoring fibrils. J Am Acad Dermatol 1994;31:884-8.
9. Vodegel RM, Jonkman MF, Pas HH, de Jong MC. U-serrated immunodeposition pattern differentiates type VII collagen targeting bullous diseases from other subepidermal bullous autoimmune diseases. Br J Dermatol 2004;151:112-8.
10. Buijsrogge JJA, Diercks GFH, Pas HH, Jonkman MF. The many faces of epidermolysis bullosa acquisita after serration pattern analysis by direct immunofluorescence microscopy. Br J Dermatol 2011;165:92-8.
11. Gammon WR, Briggaman RA, Woodley DT, Heald PW, Wheeler CE Jr. Epidermolysis bullosa acquisita—a pemphigoid-like disease. J Am Acad Dermatol 1984;11:820-32.
12. Burgeson RE. Type VII collagen, anchoring fibrils, and epidermolysis bullosa. J Invest Dermatol 1993;101:252-5.
13. Keene DR, Sakai LY, Bachinger HP, Burgeson RE. Type III collagen can be present on banded collagen fibrils regardless of fibril diameter. J Cell Biol 1987;105:2393-402.
14. Lapiere JC, Woodley DT, Parente MG, Iwasaki T, Wynn KC, Christiano AM, et al. Epitope mapping of type VII collagen. Identification of discrete peptide sequences recognized by sera from patients with acquired epidermolysis bullosa. J Clin Invest 1993;92:1831-9.
15. Gammon WR, Murrell DF, Jenison MW, Padilla KM, Prisayanh PS, Jones DA, et al. Autoantibodies to type VII collagen recognize epitopes in a fibronectin-like region of the noncollagenous (NC1) domain. J Invest Dermatol 1993;100:618-22.
16. Tanaka T, Furukawa F, Imamura S. Epitope mapping for epidermolysis bullosa acquisita autoantibody by molecularly cloned cDNA for type VII collagen. J Invest Dermatol 1994;102:706-9.
17. Jones DA, Hunt SW III, Prisayanh PS, Briggaman RA, Gammon WR. Immunodominant autoepitopes of type VII collagen are short, paired peptide sequences within the fibronectin type III homology region of the noncollagenous (NC1) domain. J Invest Dermatol 1995;104:231-5.
18. Chen M, Chan LS, Cai X, O'Toole EA, Sample JC, Woodley DT. Development of an ELISA for rapid detection of anti-type VII collagen autoantibodies in epidermolysis bullosa acquisita. J Invest Dermatol 1997;108:68-72.
19. Schmidt E, Zillikens D. Modern diagnosis of autoimmune blistering skin diseases. Autoimmun Rev 2010;10:84-9.

20. Muller R, Dahler C, Mobs C, Wenzel E, Eming R, Messer G, et al. T and B cells target identical regions of the non-collagenous domain 1 of type VII collagen in epidermolysis bullosa acquisita. Clin Immunol 2010;135:99-107.

21. Saleh MA, Ishii K, Kim YJ, Murakami A, Ishii N, Hashimoto T, et al. 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;62:169-75.

22. Schmidt E, Hopfner B, Chen M, Kuhn C, Weber L, Brocker EB, et al. Childhood epidermolysis bullosa acquisita: a novel variant with reactivity to all three structural domains of type VII collagen. Br J Dermatol 2002;147:592-7.

23. Zillikens D, Kawahara Y, Ishiko A, Shimizu H, Mayer J, Rank CV, et al. A novel subepidermal blistering disease with autoantibodies to a 200-kDa antigen of the basement membrane zone. J Invest Dermatol 1996;106:465-70.

24. Lazarova Z, Sitaru C, Zillikens D, Yancey KB. Comparative analysis of methods for detection of anti-laminin 5 autoantibodies in patients with anti-epiligrin cicatricial pemphigoid. J Am Acad Dermatol 2004;51:886-92.

25. Groth S, Recke A, Vafia K, Ludwig RJ, Hashimoto T, Zillikens D, et al. Development of a simple enzyme-linked immunosorbent assay for the detection of autoantibodies in anti-p200 pemphigoid. Br J Dermatol 2011;164:76-82.

26. Schmidt E, Dähnrich C, Rosemann A, Probst C, Komorowski L, Saschenbrecker S, et al. Novel ELISA systems for antibodies to desmoglein 1 and 3: correlation of disease activity with serum autoantibody levels in individual pemphigus patients. Exp Dermatol 2010;19:458-63.

27. Hundorfean G, Neurath MF, Sitaru C. Autoimmunity against type VII collagen in inflammatory bowel disease. J Cell Mol Med 2010;14:2393-403.

28. Terra JB, Pas HH, Hertl M, Dikkers FG, Kamminga N, Jonkman MF. IF serration pattern analysis as a diagnostic criterion in anti-laminin-332 mucous membrane pemphigoid---immuno-pathological findings and clinical experience in 10 Dutch patients. Br J Dermatol 2011;165:815-22.

29. Chen M, Doostan A, Bandyopadhyay P, Remington J, Wang X, Hou Y, et al. The cartilage matrix protein subdomain of type VII collagen is pathogenic for epidermolysis bullosa acquisita. Am J Pathol 2007;170:2009-18.

30. Tanaka H, Ishida-Yamamoto A, Hashimoto T, Hiramoto K, Harada T, Kawachi Y, et al. A novel variant of acquired epidermolysis bullosa with autoantibodies against the central triple-helical domain of type VII collagen. Lab Invest 1997;77:623-32.

31. Chen M, Keene DR, Costa FK, Tahk SH, Woodley DT. The carboxyl terminus of type VII collagen mediates antiparallel dimer formation and constitutes a new antigenic epitope for epidermolysis Bullosa acquisita autoantibodies. J Biol Chem 2001;276:21649-55.

32. Fukumoto T, Umekawa T, Higuchi M, Hashimoto T, Shumann H, Bruckner-Tuderman L, et al. Childhood epidermolysis bullosa acquisita with autoantibodies against all 3 structural domains of type VII collagen. J Am Acad Dermatol 2004;50:480-2.

33. Ishii N, Yoshida M, Ishida-Yamamoto A, Fritsch A, Elfert S, Bruckner-Tuderman L, et al. Some epidermolysis bullosa acquisita sera react with epitopes within the triple-helical collagenous domain as indicated by immunoelectron microscopy. Br J Dermatol 2009;160:1090-3.

ORIGINAL ARTICLE

Serum levels of anti-type VII collagen antibodies detected by enzyme-linked immunosorbent assay in patients with epidermolysis bullosa acquisita are correlated with the severity of skin lesions

J.H. Kim,[†] Y.H. Kim,[‡] S. Kim,[§] E.B. Noh,[†] S-E. Kim,[†] A. Vorobyev,[¶] E. Schmidt,[¶] D. Zillikens,[¶] S-C. Kim[†,*]

[†]Department of Dermatology and Cutaneous Biology Research Institute, Yonsei University College of Medicine, Seoul, Korea
[‡]INC Research, Wilmington, NC, USA
[§]Department of Laboratory Medicine, Yonsei University College of Medicine, Seoul, Korea
[¶]Department of Dermatology, University of Lübeck, Lübeck, Germany
[*]Correspondence: S.-C. Kim. E-mail: kimsc@yuhs.ac

Abstract

Background Epidermolysis bullosa acquisita (EBA) is a chronic autoimmune subepidermal bullous disease characterized by circulating autoantibodies against type VII collagen. Detecting these autoantibodies is crucial for the diagnosis of this disease, and is also useful for measuring disease activity. Enzyme-linked immunosorbent assay (ELISA), a quantitative method to measure anti-type VII collagen antibody levels, is currently available to diagnose EBA.

Objective The aim of this study was to investigate the relationship of ELISA with overall clinical severity.

Methods Sera from patients with EBA ($n = 30$), bullous pemphigoid ($n = 20$), anti-laminin $\gamma1$ pemphigoid ($n = 9$) and healthy donors ($n = 24$) were tested using ELISA, using the recombinant non-collagenous 1 (NC1) and 2 (NC2) domains of type VII collagen. Relationships between clinical characteristics, indirect immunofluoroscence (IIF) titres and ELISA values were investigated.

Results The sensitivity and specificity of the EBA ELISA were 96.7% and 98.1%, respectively. There was no significant difference between ELISA results for classic and inflammatory types. The severity of skin involvement was positively correlated with both ELISA value ($r = 0.87$, $P < 0.01$) and IIF titre ($r = 0.59$, $P < 0.01$). Time sequence analysis in four patients with EBA showed that ELISA values reflect disease activity better than IIF titres.

Conclusions Type VII collagen ELISA using the NC1 and NC2 domains is useful for diagnosing EBA and monitoring disease severity.

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Introduction

Epidermolysis bullosa acquisita (EBA) is a chronic subepidermal bullous disease characterized by autoantibodies against type VII collagen, which is the major component of the anchoring fibrils that connect the basement membrane zone to the papillary dermis.[1,2] Clinically, the two main clinical types of EBA are characterized by classic mechanobullous and inflammatory vesiculobullous features. Mechanobullous EBA manifests as trauma-induced blistering, skin fragility, scarring and milia, whereas inflammatory EBA manifests as non-traumatic blisters that mimic other bullous diseases, including bullous pemphigoid (BP), mucous membrane pemphigoid (MMP) and linear IgA dermatosis (LAD).[3]

Type VII collagen consists of a triple-helical domain flanked by a large 145 kDa non-collagenous amino-terminal (NC1) domain and a small 34 kDa non-collagenous carboxyl-terminal (NC2)

domain.[4–6] Most EBA autoantibodies recognize epitopes within the NC1 domain of type VII collagen,[7] whereas a small number of EBA autoantibodies recognize the NC2 and collagenous domains.[8–11]

The routine screening test for diagnosing EBA is direct and indirect immunofluorescence (DIF and IIF) microscopy. Particularly, salt-split IIF in immunofluorescence can distinguish EBA from BP. Patients with EBA have immune deposits on the dermal side of salt-split skin, whereas in BP, deposits are on the epidermal side. This technique, however, does not distinguish EBA from anti-laminin 332 MMP or anti-laminin γ1 pemphigoid, as these diseases also show immune deposition on the dermal side of salt-split skin. Serration pattern analysis in DIF helps to differentiate EBA from other subepidermal bullous disease.[12] EBA sera recognize the 290 kDa type VII collagen protein in immunoblotting studies. This reactivity, in fact, confirms the diagnosis of EBA; however, immunoblotting studies are practically difficult because they are time consuming and technically demanding. To overcome these problems, anti-type VII collagen enzyme-linked immunosorbent assays (ELISA) systems were developed.

As ELISA was first introduced as a diagnostic tool of pemphigus,[13,14] many ELISA studies have been conducted to diagnose various autoimmune bullous diseases, including BP (BP 180 and 230),[15,16] paraneoplastic pemphigus (envoplakin and periplakin),[17] dermatitis herpetiformis (epidermal and tissue transglutaminase)[18,19] and anti-laminin γ1 pemphigoid (laminin γ1).[20] Previous studies demonstrated that ELISAs for detecting autoantibodies in pemphigus and BP are more sensitive than immunoblotting and more related to disease activity than IIF.[21–25] ELISAs were also developed to detect autoantibodies in EBA using different recombinant proteins of the NC1 domain of type VII collagen.[26–29] Recently, recombinant NC1 and NC2 domains of type VII collagen were also used in a commercial ELISA for EBA.[30] Previous reports demonstrated that EBA ELISAs showed high sensitivity and specificity; however, only a limited number of cases have so far been reported regarding the relationship between ELISA values and disease activity. Moreover, the relationship between titres of EBA ELISA and overall clinical scores has not been characterized. In this study, we investigated the usefulness of EBA ELISA using the recombinant NC1 and NC2 domains of type VII collagen to diagnose EBA and measure disease activity.

Materials and methods

Patients and controls

Before beginning treatment, we obtained serum samples from 30 EBA patients showing (1) blisters and erosions on the skin and/or mucosa; (2) subepidermal blister formation by histopathology; (3) linear deposits of IgG autoantibodies along the dermal-epidermal junction using DIF; (4) circulating IgG autoantibodies on the dermal side of 1 mol/L salt-split skin using IIF and (5) reactivity

against the 290 kDa protein by immunoblotting using dermal extract or recombinant NC1 protein. The sera were also tested for laminin 332 and laminin γ1 immunoreactivity using immunoblotting to rule out other subepidermal bullous diseases that react to the dermal side of salt-split skin using IIF.[20,31] Furthermore, we performed IIF using HEK 293 cells transfected with the NC1 recombinant domain (Euroimmun, Lübeck, Germany). Sera from healthy blood donors were used as controls (n = 24). Additional controls included patients with BP (n = 20) and anti-laminin γ1 pemphigoid (n = 9).

A retrospective medical record review was performed on 24 EBA patients. We evaluated gender, age of onset, clinical type, oral mucosal involvement, IIF titre, methylprednisolone (MPD) dose and disease severity score (Table 1). We further assessed ELISA values and IIF titres over a time course from four patients to investigate their sequential correlation with disease severity.

Clinical severity assessment and remission

Disease severity was evaluated based on retrospective chart reviews and photographs. We modified the pemphigus disease area index (PDAI) by assessing the cutaneous and mucosal disease extent before beginning treatment. The modified PDAI score is a continuous scale ranging from 0 to 40 (Table 2). Changes in skin involvement of one patient who had seven serial sera were measured using a four-point scoring system: grade 0, quiescent (no lesion) status; grade 1, 0–10% skin involvement; grade 2, 10–30% skin involvement; grade 3, 30–60% skin involvement and grade 4, over 60% skin involvement. We defined remission as the presence of transient new lesions that heal within 1 week with minimal therapy for at least 2 months. Minimal therapy was defined as less than or equal to 8 mg/day of MPD with or without adjuvant therapy, including dapsone and colchicine.[32]

ELISA for detecting antibodies against type VII collagen

To measure the levels of antibodies against type VII collagen, we used the EBA ELISA kit (MBL, Nagoya, Japan). Two purified recombinant antigens (NC1 and NC2) were combined and coated in the same well of an ELISA microplate. The antibody titre was measured according to the manufacturer's instructions. The following formula was used to compare the index values: Index (units per millilitre of serum) = (OD of tested serum − OD of negative control)/(OD of positive control − OD of negative control) × 100. The pre-determined cut-off value by manufacturer for anti-type VII collagen antibodies was 6.14 U/mL. Sera were tested in duplicate and all steps were carried out at room temperature.

Statistical analysis

Continuous variables were summarized with median (range) or mean (standard deviation) based on the distribution. The Wilcoxon rank-sum test was performed for two group comparisons and Spearman correlation (r) was used to investigate asso-

Table 1 Patient characteristics

No.	Gender/ Age (y)	Clinical type	Oral mucosal involvement	Initial MPD dose (mg/d)	Other IS drugs (mg/d)	Severity score	ELISA (U/mL)	NC1-transfected HEK cell
1	F/73	Inflammatory (BP-like)	Positive	8	None	31	70.2	Positive
2	F/67	Inflammatory (MMP-like)	Positive	8	None	10	25.2	Positive
3	F/38	Inflammatory (BP-like)	Positive	8	None	17	22.7	Positive
4	M/49	Mechanobullous	Positive	16	None	30	65.5	Positive
5	F/46	Inflammatory (BP-like)	Positive	72	MMF 150 mg	28	41	Positive
6	M/40	Mechanobullous	Positive	20	CsA 100 mg	26	99	Positive
7	F/79	Mechanobullous	Positive	0	None	13	20	Positive
8	F/42	Mechanobullous	Positive	2	None	17	32.8	Positive
9	F/67	Mechanobullous	Positive	8	None	16	32	Positive
10	F/69	Mechanobullous	Positive	24	None	22	126.8	Positive
11	M/61	Mechanobullous	Positive	32	None	18	95.8	Positive
12	F/68	Inflammatory (BP-like)	Positive	8	None	30	133.5	Positive
13	M/74	Mechanobullous	Negative	8	None	12	3	Negative
14	M/56	Inflammatory (BP-like)	Positive	8	None	22	37.5	Positive
15	M/41	Mechanobullous	Positive	8	None	38	152.9	Positive
16	F/68	Mechanobullous	Positive	16	None	38	78.3	Positive
17	F/59	Inflammatory (BP-like)	Positive	16	CsA 100 mg	30	55	Positive
18	M/44	Inflammatory (BP-like)	Positive	16	None	11	14.4	Positive
19	F/71	Inflammatory (BP-like)	Positive	16	None	14	63.8	Positive
20	F/68	Mechanobullous	Positive	8	None	26	105.9	Positive
21	F/76	Mechanobullous	Positive	10	Rituximab 1 cycle	27	140.8	Positive
22	F/54	Mechanobullous	Negative	8	Rituximab 1 cycle	21	45.2	Positive
23	F/74	Mechanobullous	Positive	8	None	17	25.3	Positive
24	F/83	Mechanobullous	Positive	8	None	9	24.6	Positive

BP, bullous pemphigoid; MMP, mucous membrane pemphigoid; MPD, methylprednisolone; IS, immunosuppressive; MMF, mycophenolate mofetil; CsA, cyclosporine A

Table 2 Modified Pemphigus Disease Area Index (PDAI) for patients with epidermolysis bullosa acquisita

| Anatomic location	Erosion/blisters or new erythema
	\<Skin> **0 Absent** **1 1–3 lesions, none >5 cm diameter** **2 >3 lesions, none >5 cm diameter** **3 >3 lesions, and/or at least one >5 cm diameter** **\<Mucosa>** **0 Absent** **5 1–3 lesions** **10 >3 lesions**
Scalp	/3
Face	/3
Neck	/3
Chest	/3
Abdomen	/3
Back, buttocks	/3
Arms	/3
Hands	/3
Legs	/3
Feet	/3
Mucosa	/10
Total points	**/40**

ciations. Statistical analyses were performed using SAS 9.2 and R 2.13.0 under the alpha level of 0.05.

Results

ELISA using recombinant NC1 and NC2 proteins of type VII collagen

A total of 30 EBA patients who presented at the Department of Dermatology in Gangnam Severance Hospital between April 1993 and June 2011 were included in this study. The mean (SD) age of EBA patients was 57.9 (15.4) years. Twenty nine of 30 sera had positive ELISA results. One of the sera from the control group showed ELISA values beyond the cut-off value (6.14 U/mL). The sensitivity and specificity of the ELISA were 96.7% and 98.1%, respectively (Fig. 1). IIF using HEK 293 cells transfected with NC1 were also 96.7% positive.

To assess the relationship between clinical features and ELISA values, we divided EBA patients into mechanobullous and inflammatory type (Table 1). The median ELISA values of the fifteen mechanobullous type and nine inflammatory type EBA patients were 65.5 (3.0, 152.9) and 31.4 (0.9, 149.3), respectively. The ELISA values for mechanobullous and inflammatory type EBA were not significantly different ($P = 0.15$).
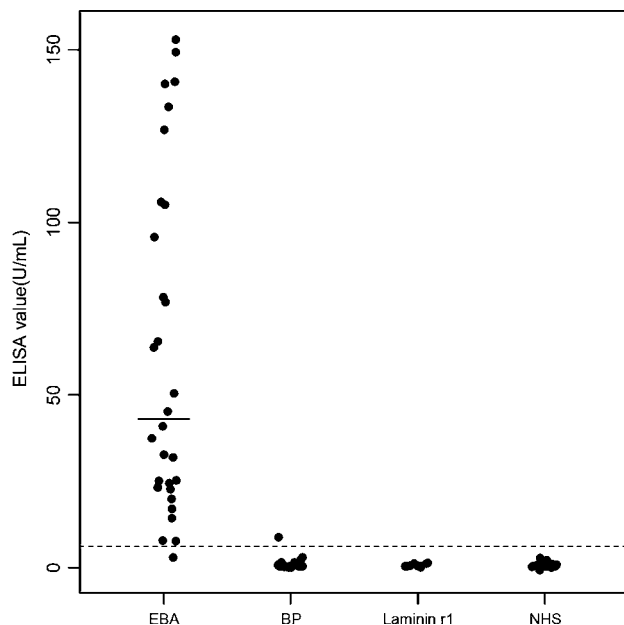
Figure 1 ELISA values in serum samples from patients with epidermolysis bullosa acquisita (EBA), bullous pemphigoid (BP), anti-laminin γ1 pemphigoid (laminin γ1) and healthy blood donors (NHS). The sensitivity and specificity of the EBA ELISA system were 96.7% and 98.1%, respectively. The dashed line indicates the cut-off value (6.14 U/mL) and the solid line indicates the median value (41.3 U/mL).

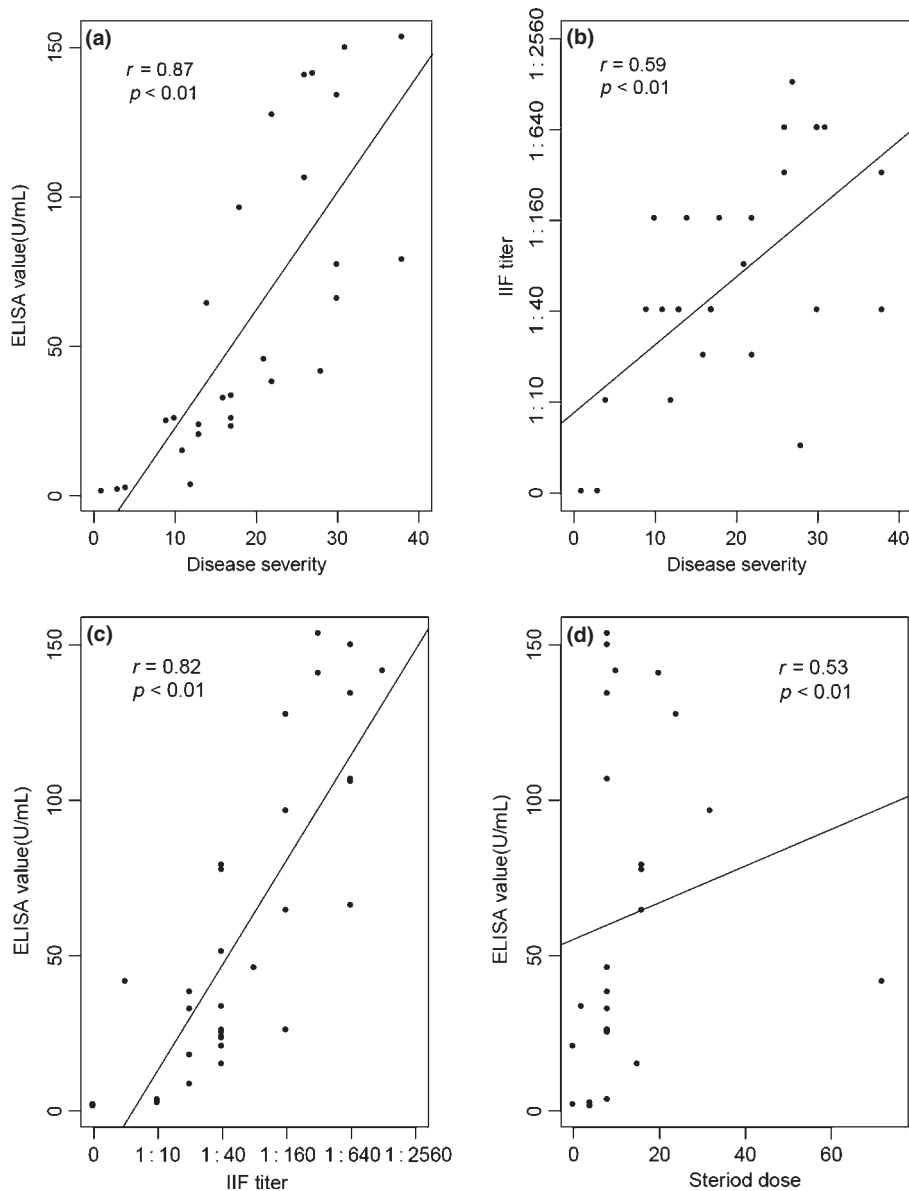### Correlation between index values and disease severity

Disease severity was positively correlated with both ELISA values and IIF titres ($r = 0.87$, $P < 0.01$ and $r = 0.59$, $P < 0.01$). Only ELISA values were correlated with the initial corticosteroid dose ($r = 0.53$, $P < 0.01$). There was a correlation between ELISA values and IIF titres ($r = 0.82$, $P < 0.01$) (Fig. 2).

Disease progression was compared with the IIF titres and ELISA values to investigate, which is more closely related to the disease course. First, ELISA values and IIF titres from three patients were measured before treatment and after remission (Fig. 3a,b, respectively). ELISA values declined in all patients after remission, whereas IIF titre increased in one patient after remission. Second, we performed a retrospective chart review of another patient who had sera collected serially. A 40-year-old man, who had blisters and erosions on the whole body including oral mucosa, has been treated with prednisolone (10–25 mg/d), colchicine (1.2 mg/d) and dapsone (100–150 mg/d) based on his disease severity. When the prednisolone was tapered to 10 mg, he experienced an active flare. ELISA and IIF tests were performed seven times during 10 years of follow-up. We found that ELISA values are better correlated with disease activity than IIF titres (Fig. 4).

Discussion

Although the pathomechanism of blister formation in EBA remains unclear, several studies have provided evidence that the autoantibodies against type VII collagen are pathogenic. When rabbit and human antibodies specific to the NC1 domain of type VII collagen were passively transferred to mice, they induced subepidermal blister formation and nail dystrophy mimicking EBA.[33,34] The active mouse model of EBA was established by immunizing mice with the recombinant NC1 domain of murine type VII collagen.[35,36] In addition, a transient neonate EBA resulted from a vertical transfer of maternal IgG autoantibodies against type VII collagen from a mother with EBA, suggesting that these transmitted autoantibodies induced blister formation in the neonate.[37] In addition, serial titres of anti-type VII collagen IgG from each patient with EBA reflect disease activity.[26,30,38] Although previously, levels of autoantibodies to type VII collagen, detected using ELISA, have been shown to correlate with disease activity at different time points in individual patients, we herein demonstrate a correlation of autoantibody serum levels with overall disease activity in a large cohort of EBA patients.

Different ELISAs for the detection of autoantibodies in EBA have been developed using recombinant type VII collagen. Chen et al.[28] reported 100% reactivity by an ELISA using eukaryote-derived recombinant NC1 domain of type VII collagen. In another study of EBA, ELISA using baculovirus-derived recombinant NC1 had 67% sensitivity and 100% specificity.[26] Recently, Komorowski et al.[29] yielded 94.5% sensitivity and 98.7% specificity by an ELISA using recombinant NC1 expressed in human HEK 293 cells. In this study, an ELISA against recombinant NC1 and NC2 domains showed

Figure 2 The relationships between (a) ELISA value or (b) indirect immunofluorescence (IIF) titre and disease severity score. ELISA values ($r = 0.87$, $P < 0.01$) and IIF titres ($r = 0.59$, $P < 0.01$) correlated with disease severity scores. (c) ELISA values was also correlated with IIF titres ($r = 0.82$, $P < 0.01$). (d) ELISA values correlated with initial methylprednisolone (MPD) dose ($r = 0.53$, $P < 0.01$), but IIF titres did not.

high sensitivity (96.7%) and specificity (98.1%). Our findings are comparable with those of a previous study reporting 91.8% sensitivity and 98.1% specificity using the same ELISA system.[30] IIF using HEK 293 cells transfected with NC1 showed similar sensitivity, which means that almost all sera in this study have autoantibodies against NC1 domain of type VII collagen. Nevertheless, it would be advisable to use EBA ELISA containing recombinant NC1 and NC2 domains rather than using recombinant NC1

domain only, as some EBA patients have autoantibodies against NC2 domain only.[8,9] IIF titre, ELISA value, initial MPD dose and disease severity score were not significantly different between mechanobullous and inflammatory type EBA (data not shown).

Both EBA ELISA and IIF values are positively correlated with disease severity scores. In our study, three aspects suggest that ELISA results better correlate with disease activity than IIF. First,
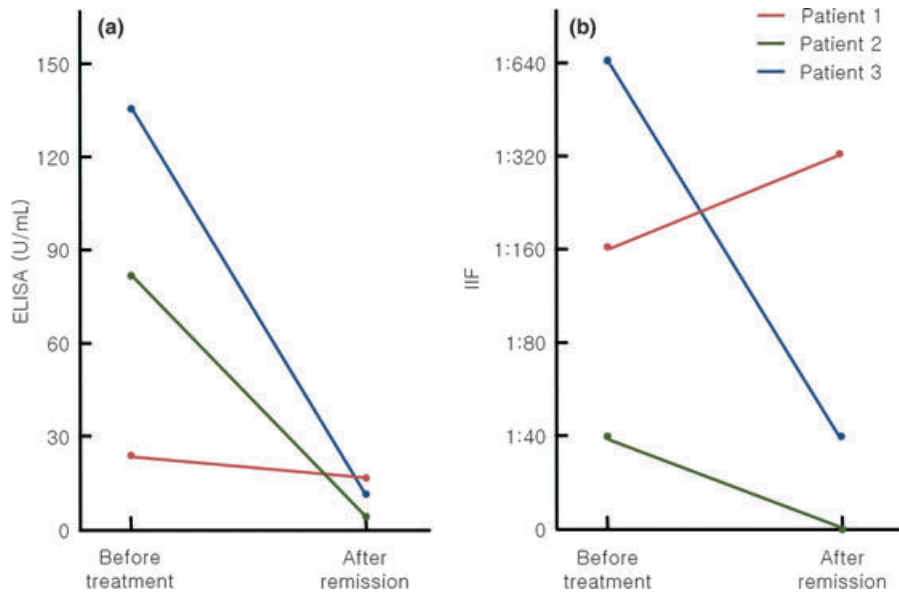
Figure 3 Indirect immunofluorescence (IIF) titres (a) and ELISA values (b) of three patients before treatment and after remission.
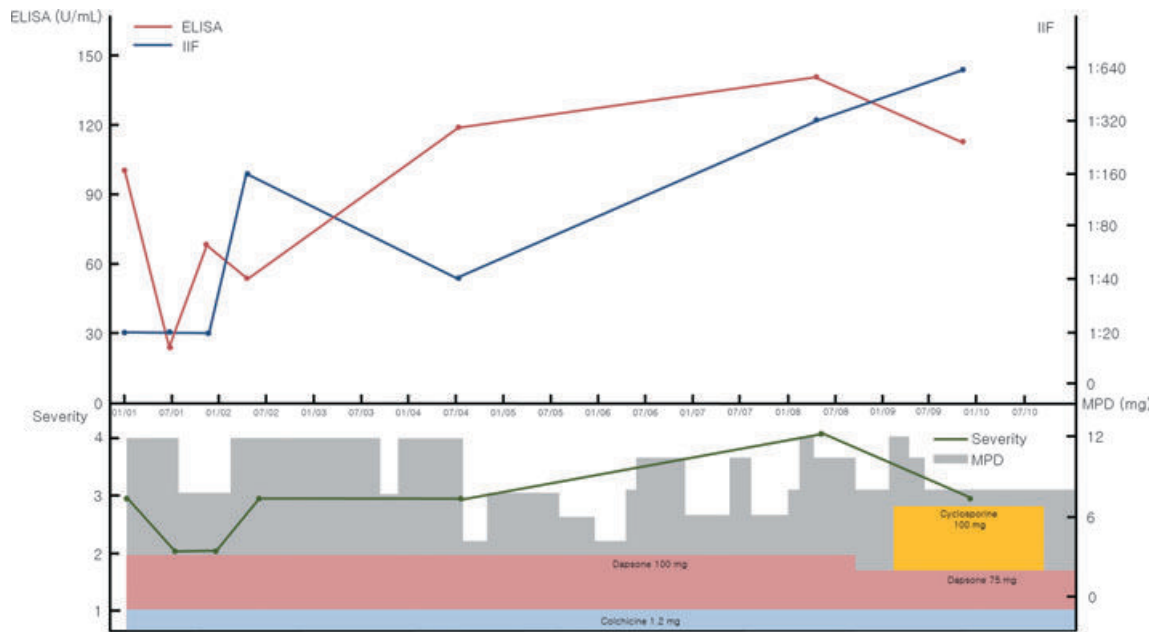
Figure 4 Clinical course, methylprednisolone (MPD) dose, indirect immunofluorescence (IIF) titres and ELISA values from January 2001 to January 2011 of one patient with epidermolysis bullosa acquisita.

we observed that only ELISA, not IIF, correlated with the initial steroid dose, which usually reflects EBA severity.[39] Second, we compared ELISA values and IIF titres from three patients with EBA before treatment and after remission. The ELISA values for all three patients declined after remission, whereas one patient's IIF titre increased after remission (Fig. 3). Finally, sera

collected from one patient at seven different times also showed that ELISA values better represent disease severity than IIF titres (Fig. 4).

In conclusion, the ELISA using recombinant NC1 and NC2 proteins is useful for the diagnosis of EBA and evaluation of disease severity.

References

1 Roenigk HH Jr, Ryan JG, Bergfeld WF. Epidermolysis bullosa acquisita. Report of three cases and review of all published cases. *Arch Dermatol* 1971; **103**: 1–10.

2 Woodley DT, Briggaman RA, O'Keefe EJ *et al.* Identification of the skin basement-membrane autoantigen in epidermolysis bullosa acquisita. *N Engl J Med* 1984; **310**: 1007–1013.

3 Kim JH, Kim YH, Kim SC. Epidermolysis bullosa acquisita: a retrospective clinical analysis of 30 cases. *Acta Derm Venereol* 2011; **91**: 307–312.

4 Chung HJ, Uitto J. Type VII collagen: the anchoring fibril protein at fault in dystrophic epidermolysis bullosa. *Dermatol Clin* 2010; **28**: 93–105.

5 Christiano AM, Rosenbaum LM, Chung-Honet LC *et al.* The large non-collagenous domain (NC-1) of type VII collagen is amino-terminal and chimeric. Homology to cartilage matrix protein, the type III domains of fibronectin and the A domains of von Willebrand factor. *Hum Mol Genet* 1992; **1**: 475–481.

6 Greenspan DS. The carboxyl-terminal half of type VII collagen, including the non-collagenous NC-2 domain and intron/exon organization of the corresponding region of the COL7A1 gene. *Hum Mol Genet* 1993; **2**: 273–278.

7 Lapiere JC, Woodley DT, Parente MG *et al.* Epitope mapping of type VII collagen. Identification of discrete peptide sequences recognized by sera from patients with acquired epidermolysis bullosa. *J Clin Invest* 1993; **92**: 1831–1839.

8 Chen M, Keene DR, Costa FK *et al.* The carboxyl terminus of type VII collagen mediates antiparallel dimer formation and constitutes a new antigenic epitope for epidermolysis Bullosa acquisita autoantibodies. *J Biol Chem* 2001; **276**: 21649–21655.

9 Ishii N, Yoshida M, Hisamatsu Y *et al.* 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* 2004; **150**: 843–851.

10 Tanaka H, Ishida Yamamoto A, Hashimoto T *et al.* A novel variant of acquired epidermolysis bullosa with autoantibodies against the central triple-helical domain of type VII collagen. *Lab Invest* 1997; **77**: 623–632.

11 Ishii N, Yoshida M, Ishida-Yamamoto A *et al.* Some epidermolysis bullosa acquisita sera react with epitopes within the triple-helical collagenous domain as indicated by immunoelectron microscopy. *Br J Dermatol* 2009; **160**: 1090–1093.

12 Buijsrogge JJ, Diercks GF, Pas HH *et al.* The many faces of epidermolysis bullosa acquisita after serration pattern analysis by direct immunofluorescence microscopy. *Br J Dermatol* 2011; **165**: 92–98.

13 Amagai M, Hashimoto T, Shimizu N *et al.* Absorption of pathogenic autoantibodies by the extracellular domain of pemphigus vulgaris antigen (Dsg3) produced by baculovirus. *J Clin Invest* 1994; **94**: 59–67.

14 Ishii K, Amagai M, Hall RP *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.

15 Kromminga A, Sitaru C, Hagel C *et al.* Development of an ELISA for the detection of autoantibodies to BP230. *Clin Immunol* 2004; **111**: 146–152.

16 Zillikens D, Mascaro JM, Rose PA *et al.* A highly sensitive enzyme-linked immunosorbent assay for the detection of circulating anti-BP180 autoantibodies in patients with bullous pemphigoid. *J Invest Dermatol* 1997; **109**: 679–683.

17 Probst C, Schlumberger W, Stocker W *et al.* Development of ELISA for the specific determination of autoantibodies against envoplakin and periplakin in paraneoplastic pemphigus. *Clin Chim Acta* 2009; **410**: 13–18.

18 Rose C, Armbruster FP, Ruppert J *et al.* Autoantibodies against epidermal transglutaminase are a sensitive diagnostic marker in patients with dermatitis herpetiformis on a normal or gluten-free diet. *J Am Acad Dermatol* 2009; **61**: 39–43.

19 Dieterich W, Laag E, Bruckner Tuderman L *et al.* Antibodies to tissue transglutaminase as serologic markers in patients with dermatitis herpetiformis. *J Invest Dermatol* 1999; **113**: 133–136.

20 Groth S, Recke A, Vafia K *et al.* Development of a simple enzyme-linked immunosorbent assay for the detection of autoantibodies in anti-p200 pemphigoid. *Br J Dermatol* 2011; **164**: 76–82.

21 Lehman JS, Camilleri MJ, Gibson LE. Epidermolysis bullosa acquisita: concise review and practical considerations. *Int J Dermatol* 2009; **48**: 227–235. quiz 35–36.

22 Leuci S, Gurcan HM, Ahmed AR. Serological studies in bullous pemphigoid: a literature review of antibody titers at presentation and in clinical remission. *Acta Derm Venereol* 2010; **90**: 115–121.

23 Lee EH, Kim YH, Kim S. Usefulness of enzyme-linked immunosorbent assay using recombinant BP180 and BP230 for serodiagnosis and monitoring disease activity of bullous pemphigoid. *Ann Dermatol* 2012; **24**: 45–55.

24 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.

25 Schmidt E, Obe K, Brocker EB *et al.* Serum levels of autoantibodies to BP180 correlate with disease activity in patients with bullous pemphigoid. *Arch Dermatol* 2000; **136**: 174–178.

26 Muller R, Dahler C, Mobs C *et al.* T and B cells target identical regions of the non-collagenous domain 1 of type VII collagen in epidermolysis bullosa acquisita. *Clin Immunol* 2010; **135**: 99–107.

27 Chen M, Doostan A, Bandyopadhyay P *et al.* The cartilage matrix protein subdomain of type VII collagen is pathogenic for epidermolysis bullosa acquisita. *Am J Pathol* 2007; **170**: 2009–2018.

28 Chen M, Chan LS, Cai X *et al.* Development of an ELISA for rapid detection of anti-type VII collagen autoantibodies in epidermolysis bullosa acquisita. *J Invest Dermatol* 1997; **108**: 68–72.

29 Komorowski L, Muller R, Vorobyev A *et al.* Sensitive and specific assays for routine serological diagnosis of epidermolysis bullosa acquisita. *J Am Acad Dermatol* 2012; doi: 10.1016/j.jaad.2011.12.032. [Epub ahead of print].

30 Saleh MA, Ishii K, Kim YJ *et al.* 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; **62**: 169–175.

31 Rose C, Schmidt E, Kerstan A *et al.* Histopathology of anti-laminin 5 mucous membrane pemphigoid. *J Am Acad Dermatol* 2009; **61**: 433–440.

32 Kim J.H., Kim YH, Kim SC. Epidermolysis bullosa acquisita: a retrospective clinical analysis of 30 cases. *Acta Derm Venereol* 2011; **91**: 307–312.

33 Sitaru C, Mihai S, Otto C *et al.* Induction of dermal-epidermal separation in mice by passive transfer of antibodies specific to type VII collagen. *J Clin Invest* 2005; **115**: 870–878.

34 Woodley DT, Ram R, Doostan A *et al.* Induction of epidermolysis bullosa acquisita in mice by passive transfer of autoantibodies from patients. *J Invest Dermatol* 2006; **126**: 1323–1330.

35 Ludwig RJ, Recke A, Bieber K *et al.* Generation of antibodies of distinct subclasses and specificity is linked to H2s in an active mouse model of epidermolysis bullosa acquisita. *J Invest Dermatol* 2011; **131**: 167–176.

36 Sitaru C, Chiriac MT, Mihai S *et al.* Induction of complement-fixing autoantibodies against type VII collagen results in subepidermal blistering in mice. *J Immunol* 2006; **177**: 3461–3468.

37 Abrams ML, Smidt A, Benjamin L *et al.* Congenital epidermolysis bullosa acquisita: vertical transfer of maternal autoantibody from mother to infant. *Arch Dermatol* 2011; **147**: 337–341.

38 Sato M, Ishitsuka A, Shibuya Y *et al.* Time-course of the change in titre of antibodies against type VII collagen in a patient with epidermolysis bullosa acquisita. *Acta Derm Venereol* 2012; doi: 10.2340/00015555-1339. [Epub ahead of print].

39 Lyakhovitsky A, Baum S, Scope A *et al.* The impact of stratifying initial dose of corticosteroids by severity of pemphigus vulgaris on long-term disease severity. *Int J Dermatol* 2011; **50**: 1014–1019.

Autoantibodies to Multiple Epitopes on the Non-Collagenous-1 Domain of Type VII Collagen Induce Blisters

Artem Vorobyev[1], Hideyuki Ujiie[2], Andreas Recke[1], Jacqueline J.A. Buijsrogge[3], Marcel F. Jonkman[3], Hendri H. Pas[3], Hiroaki Iwata[1], Takashi Hashimoto[4], Soo-Chan Kim[5], Jong Hoon Kim[5], Richard Groves[6], Unni Samavedam[1], Yask Gupta[1], Enno Schmidt[1], Detlef Zillikens[1], Hiroshi Shimizu[2] and Ralf J. Ludwig[1]

Epidermolysis bullosa acquisita (EBA) is an autoimmune blistering disease of the skin and mucous membranes, characterized by autoantibodies against type VII collagen (COL7), a major component of anchoring fibrils. Different clinical EBA phenotypes are described, including mechanobullous and inflammatory variants. Most EBA patients' sera react with epitopes located within the non-collagenous 1 (NC1) domain of human COL7. However, it has remained unclear whether antibody binding to these different epitopes is pathogenically relevant. To address this issue, we generated recombinant proteins covering the entire NC1 domain. IgG reactivity with these proteins was analyzed in sera of 69 EBA patients. Most recognized clusters of epitopes throughout the NC1 domain. No correlation was detected between antibody specificity and clinical phenotype. To study the pathogenicity of antibodies specific to different NC1 subdomains, rabbit antibodies were generated. All these antibodies caused dermal–epidermal separation ex vivo. Antibodies against two of these subdomains were injected into mice carrying null mutations of mouse COL7 and the human COL7 transgene and induced subepidermal blisters. We here document that autoantibodies to COL7, independent of the targeted epitopes, induce blisters both ex vivo and in vivo. In addition, using COL7-humanized mice, we provide in vivo evidence of pathogenicity of autoantibodies binding to human COL7.

INTRODUCTION

Autoimmune diseases are characterized by the presence of autoreactive T or B cells that mediate an uncontrolled inflammatory response and are responsible for clinical disease manifestation (Rose and Bona, 1993). Over the past decades, incidence of autoimmune diseases has constantly increased in developed countries (Bach, 2002). Epidermolysis bullosa acquisita (EBA) is a prototypical, organ-specific autoimmune disease. EBA is characterized by the presence of circulating and tissue-bound antibodies to type VII collagen (COL7), a major component of anchoring fibrils (Ludwig, 2013; Sakai et al.,

[1]Department of Dermatology, University of Lübeck, Lübeck, Germany; [2]Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; [3]Department of Dermatology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; [4]Department of Dermatology, Kurume University School of Medicine and Kurume University Institute of Cutaneous Cell Biology, Fukuoka, Japan; [5]Department of Dermatology and Cutaneous Biology Research Institute, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Korea and [6]Department of Immunodermatology, St John's Institute of Dermatology, St Thomas' Hospital, London, UK

Correspondence: Artem Vorobyev, Lübeck Institute of Experimental Dermatology (LIED), Ratzeburger Allee 160, Lübeck 23538, Germany. E-mail: artem.vorobyev@uksh.de

1986; Schmidt and Zillikens, 2013; Woodley et al., 1984; Woodley et al., 1986). Pathogenicity of anti-COL7 autoantibodies has been demonstrated in several experimental models (Ludwig et al., 2013). Clinically, different EBA phenotypes are described: (i) classical mechanobullous (Roenigk et al., 1971), and (ii) inflammatory type, including bullous pemphigoid- (Gammon et al., 1984), Brunsting-Perry pemphigoid- (Kurzhals et al., 1991), linear IgA dermatosis- (Zambruno et al., 1994), and mucous membrane pemphigoid-like variants (Dahl, 1979). Whereas events leading to blister formation in the inflammatory EBA variant are relatively well characterized, it remains unclear which processes cause blistering in mechanobullous EBA—e.g., perturbation of interactions of COL7 and other components of the dermal–epidermal junction (Gupta et al., 2012; Ishii et al., 2010; Ludwig, 2012).

Detailed analysis of the autoantibody response in EBA patients showed that, in the majority of cases, IgG anti-COL7 autoantibodies are detected. Characterization of IgG isotypes demonstrated the prevalence of IgG1 and IgG4 subclasses (Bernard et al., 1991; Oostingh et al., 2005). In addition, IgA anti-COL7 reactivity, either exclusively or in combination with IgG autoantibodies, is observed in 50–60% of patients (Buijsrogge et al., 2011; Gandhi et al., 2000; Kim et al., 2011). Yet, this understanding of the autoimmune response has not been useful to differentiate among the mechanobullous and

the inflammatory EBA variants (Gandhi *et al.*, 2000). Epitope mapping of the COL7-specific B-cell immune response in EBA patients identified the NC1 domain of COL7 as the major antigenic site (Gammon *et al.*, 1993; Jones *et al.*, 1995; Tanaka *et al.*, 1994). Binding of patient autoantibodies to the collagenous or the NC2 domain is rarely observed (Ishii *et al.*, 2004; Saleh *et al.*, 2011). Further epitope mapping of EBA patients' sera identified 4 major antigenic epitopes within the NC1 domain (Chen *et al.*, 2007; Gammon *et al.*, 1993; Gandhi *et al.*, 2000; Ishii *et al.*, 2004; Lapiere *et al.*, 1993). However, it remained unclear whether the specificities of the anti-COL7 antibodies are associated with certain clinical EBA phenotypes.

The pathogenic relevance of patient's anti-COL7 IgG has been demonstrated by its ability to induce dermal–epidermal separation when incubated with cryosections of human skin in the presence of neutrophils (Sitaru *et al.*, 2002a) and by induction of subepidermal blisters in mice, which were injected with patient antibodies, affinity-purified against the NC1 domain (Woodley *et al.*, 2006). Experimental EBA can also be induced in mice by transfer of antibodies to epitopes located within the murine sixth–ninth fibronectin-3 (FNIII)-like repeat or by immunization with the same antigen (Sitaru *et al.*, 2006; Sitaru *et al.*, 2005). Furthermore, transfer of patient autoantibodies, affinity-purified against the cartilage matrix protein (CMP) domain, also induced EBA after transfer into mice (Chen *et al.*, 2007). Recently, pathogenicity of anti-von Willebrand factor 2 (anti-vWFA2) antibodies has been demonstrated, both by transfer of autoantibodies and by immunization of mice with vWFA2 (Iwata *et al.*, 2013). Collectively, sera from EBA patients bind to many epitopes located within the NC1 domain, but pathogenicity has only been demonstrated for few of these. It remains unclear whether the broad epitope recognition observed in EBA patients is an epiphenomenon resulting from intramolecular epitope spreading or whether this broad recognition pattern is required for blister induction.

In a cohort of 69 EBA patients as well as in *ex vivo* and *in vivo* models of EBA, we therefore here addressed whether (i) the different clinical EBA phenotypes are associated with certain autoantibody specificities, and (ii) whether autoantibodies to certain domains of NC1 are capable to induce subepidermal blisters.

RESULTS

Expression of recombinant subdomains of COL7 NC1
Recombinant overlapping proteins for NC1 subdomains and NC2 domain were successfully produced in *E. coli* Rosetta DE3 (Supplementary Figure S1). CMP-FNIII1 and FNIII9-vWFA2 fragments were insoluble. After refolding, CMP-FNIII1 protein could be obtained in sufficient amounts and purity for rabbit immunization. FNIII9-vWFA2 protein purity did not allow using this protein for raising rabbit antibodies. Correct size of all proteins was confirmed by SDS-PAGE gel analysis (Supplementary Figure S1).

EBA patients' sera recognize multiple epitopes within NC1
For determination of epitope recognition patterns of patient autoantibodies, we analyzed IgG reactivity with recombinant

proteins of NC1 domain. In preliminary studies, IgG1, IgG2, IgG3, and IgG4 secondary antibodies for detection of reactivity to the recombinant fragments were evaluated for specificity. Although the use of secondary antibodies specific for IgG1, IgG2, and IgG3 showed high reactivity with normal human sera, the use of IgG4 secondary antibodies did not or only rarely show this unspecific binding (not shown). This is in line with the routine diagnosis of EBA in our laboratory, where an anti-IgG4 antibody is used for the same reason (Schmidt and Zillikens, 2013). Furthermore, here (Supplementary Table S1 online) and elsewhere (Komorowski *et al.*, 2013) IgG4 reactivity correlated well with the COL7-specific binding of other IgG to this protein. Therefore, subsequent epitope mapping studies were carried out using anti-human IgG4 as a secondary antibody. In most cases, the IgG4 reactivity pattern in sera from EBA patients was not restricted to single epitopes, but rather showed clusters of reactivity, recognizing multiple subdomains of NC1 domain (Figure 1). Detailed analysis of the number of epitopes recognized by EBA sera showed that most sera (58%) had autoantibody reactivity to two stretches (defined as a continuous recognition of subsequent recombinant proteins). The mean length of such stretches consisted on an average of 3.5 ± 2.7 recombinant proteins. Sera of 16 patients recognized a single stretch with a length of 7.2 ± 2.7 recombinant proteins; autoantibodies from 13 patients were specific to three different stretches, mean length 2.0 ± 1.5. We also generated recombinant NC2. In 41 tested sera from our cohort of EBA patients, 9 reacted to this epitope.

Gender and age, but not the clinical EBA variant, correlate with autoantibody specificity
To test whether certain epitope recognition patterns are specific for the different clinical phenotypes of EBA, we performed homogeneity distribution analysis and Pearson's correlation coefficient analysis. Interestingly, no statistically significant correlation was found between the recognized epitopes within the NC1 domain and the clinical phenotype. However, IgG4 binding pattern was dependent on age and gender of the patients (Figure 2). Younger age was associated with recognition of C-terminally located recombinant proteins, as well as NC2. Male gender was associated with recognition of FNIII2-FNIII3 and FNIII3-FNIII4 fragments. Antibodies from female EBA patients bound more to FNIII8-FNIII9 (Figure 2).
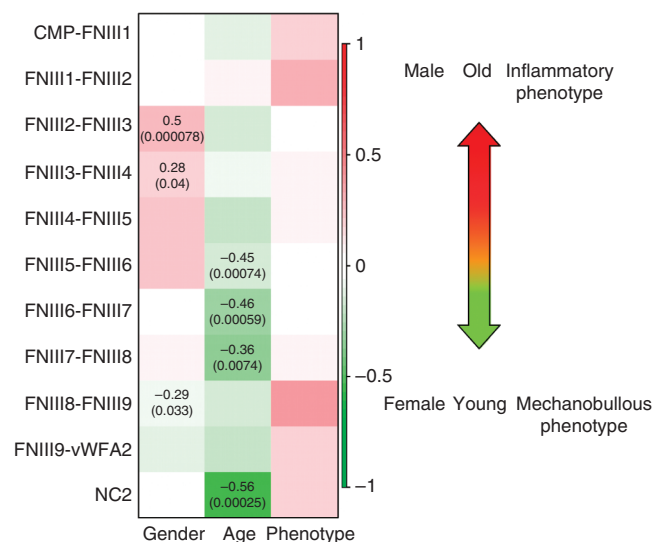
Autoantibodies against specific human NC1 subdomains show intramolecular cross-reactivity and varying cross-reactivity with murine skin
For functional studies, polyclonal rabbit anti-COL7 antibodies were generated by immunizing rabbits with all recombinant proteins spanning the human NC1 domain with the exception of FNIII9-vWFA2. Subdomains of NC1 domain of COL7 consist of the flanking CMP and vWFA2 domains and 9 FNIII-like repeats; these subdomains share some homology (Parente *et al.*, 1991). We first evaluated the possibility of intramolecular cross-reactivity of autoantibodies directed to specific domains within NC1. For this, we performed western blotting analysis using affinity-purified anti-NC1 antibodies
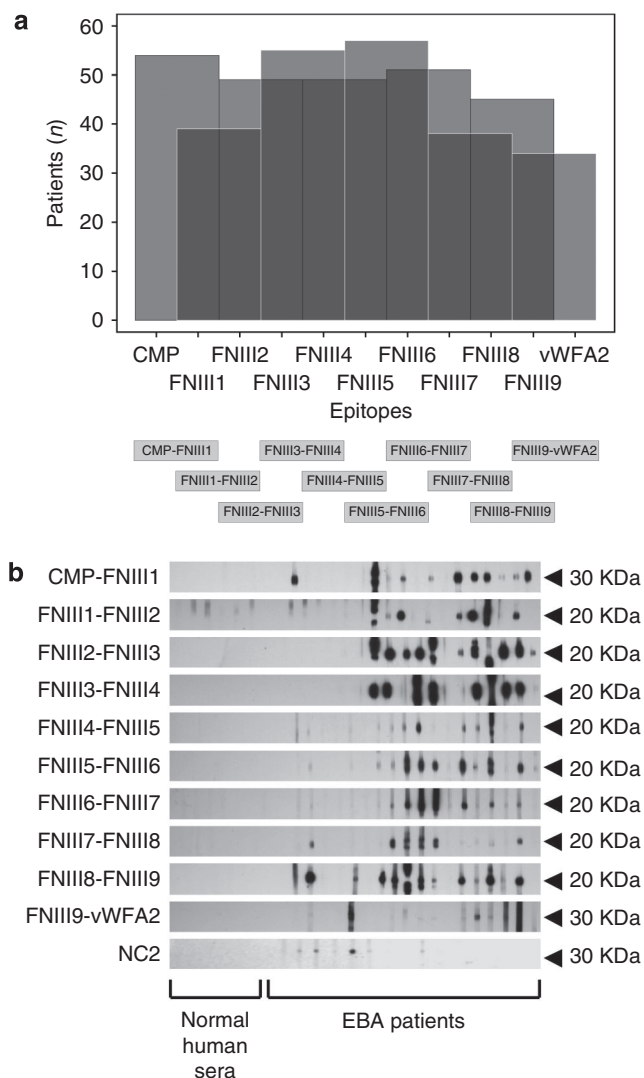
Figure 1. Epidermolysis bullosa acquisita (EBA) patients' sera recognize multiple epitopes within the non-collagenous 1 (NC1) domain. (**a**) Schematic representation of the epitope-specific reactivity of 69 EBA patients' sera tested by western blotting. The *y* axis corresponds to the number of EBA patient sera reacting with the epitopes specified on the *x* axis. (**b**) Representative images of western blotting analysis of selected EBA patients' sera. Bands show reactivity of 18 EBA sera with different recombinant proteins of human type VII collagen NC1. As negative controls, randomly selected normal human sera were used.


Figure 2. Correlation of gender, age, and clinical phenotype with autoantibody reactivity. Heatmap table showing Pearson's correlation coefficient of epitope-specific autoantibody reactivity with gender, age, and clinical phenotype. Nonsignificant values are shown as blank. Top line of the numbers in each box corresponds to Pearson's correlation coefficient and bottom line (in brackets) to *P*-value. In the left column, red and green colors correspond to male and female gender, in the middle column to older and younger age, and in the right column to inflammatory and mechanobullous phenotypes, respectively.

from rabbit sera and recombinant proteins. All antibodies showed reactivity to the respective antigen. Interestingly, for several antibodies, we observed binding to NC1 subdomains outside of the used immunogen. For example, FNIII1-FNIII2 was recognized not only by the corresponding anti-CMP-FNIII1, anti-FNIII1-FNIII2, and anti-FNIII2-FNIII3 antibodies but also by anti-FNIII4-FNIII5, anti-FNIII5-FNIII6, and anti-FNIII7-FNIII8 antibodies. (Figure 3a and b). Antibodies to NC2 were also generated. However, these did not bind to human salt-split skin (not shown). The presence of intermolecular cross-reactivity was also observed in the immunapheresis material of an EBA patient (Supplementary Figure S2A and B online).

Next, as NC1 portions of murine and human share some homology, we evaluated whether antibodies to human COL7 can also recognize murine COL7. All our polyclonal anti-COL7 antibodies bound to the dermal side of human salt-split skin. Endpoint titers ranged from 1:1,600 (anti-FNIII2-FNIII3) to 1:25,600 (anti-FNIII4-FNIII5 and anti-FNIII7-FNIII8) (Figure 3c). Using murine skin as a substrate for indirect immunofluorescence microscopy, we observed binding of most antibodies (Figure 3c). However, on murine skin, a similar endpoint titer was only observed for anti-CMP-FNIII1, whereas no binding to murine skin was observed for anti-FNIII3-FNIII4 and anti-FNIII7-FNIII8. All other antibodies showed a lower endpoint titer on murine, compared with human, skin.

### Antibodies to different epitopes of COL7 induce dermal–epidermal separation *ex vivo*
We next determined the ability of anti-COL7 autoantibodies to induce dermal–epidermal separation *ex vivo* by incubating our anti-COL7 autoantibodies with cryosections of human skin in the presence of leukocytes. Interestingly, all anti-COL7 autoantibodies recruited leukocytes to the dermal–epidermal junction and caused dermal–epidermal separation, located in lamina lucida (Figure 4, Supplementary Figure S3 online). This is in line with previous observations of intra-lamina lucida blister formation in EBA patients (Fine *et al.*, 1989). For most immune sera tested, the extent of dermal–epidermal separation correlated with antibody concentration (Figure 4). Preimmune sera neither recruited leukocytes to the dermal–epidermal junction, nor induced dermal–epidermal separation.
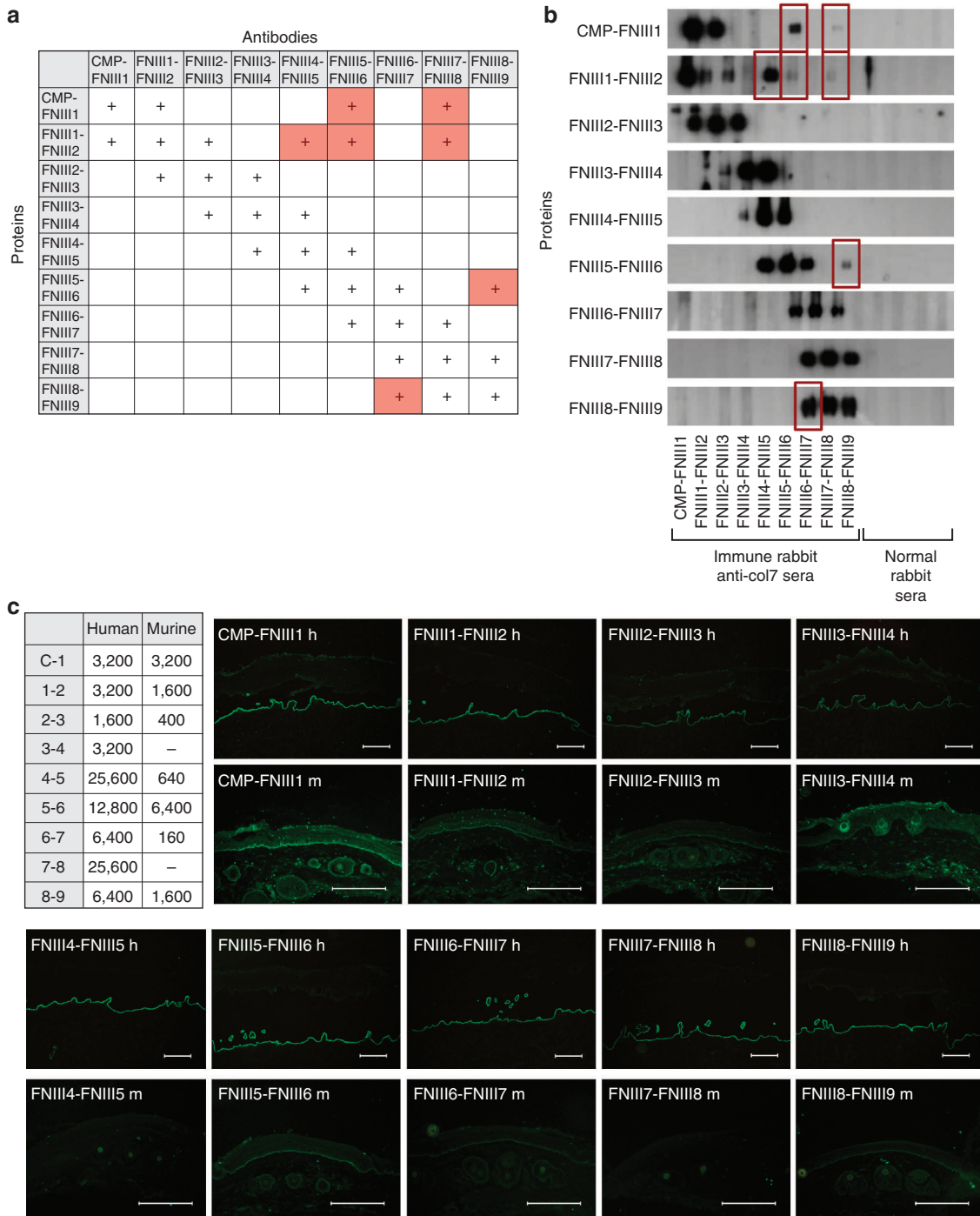
Figure 3. The presence of intramolecular cross-reactivity of antibodies to type VII collagen (COL7) non-collagenous 1 (NC1) and cross-reactivity of antibodies between human and murine skin. (**a**) Summary and (**b**) corresponding images of western blotting analyses, showing cross-reactivity of immune sera with recombinant fragments of human COL7. Marked in red is reactivity outside the antigen used for immunization. (**c**) Summary and corresponding images of end point titers of the generated rabbit immune sera, specific to different subdomains of COL7 NC1, on human 1 M salt-split skin (marked with h) and murine skin (marked with m) by indirect immunofluorescence microscopy. Bar = 100 μm. Serum dilutions used for the shown images were adjusted to a 100-fold lower dilution compared with the end point titer of each epitope-specific serum determined on human skin as substrate. For immunofluorescence images on human and murine skin, the same serum dilutions were used. The summary table on the left side of top panel shows end point titers of the subdomain-specific sera on human salt-split skin and murine skin. Abbreviations: C-1, CMP-FNIII1, 1-2, FNIII1-FNIII2, all other abbreviations correspond to this format.

To validate these findings using human sera, we affinity-purified IgG antibodies direct against the different epitopes within the NC1 domain from immunoapheresis material from one EBA patient. Here, we could isolate IgG antibodies against all NC1 fragments used within the study. The obtained material was not sufficient to be tested in the above *ex vivo* dermal–epidermal separation assay. To test for a possible neutrophil activating capability of these autoantibodies, we
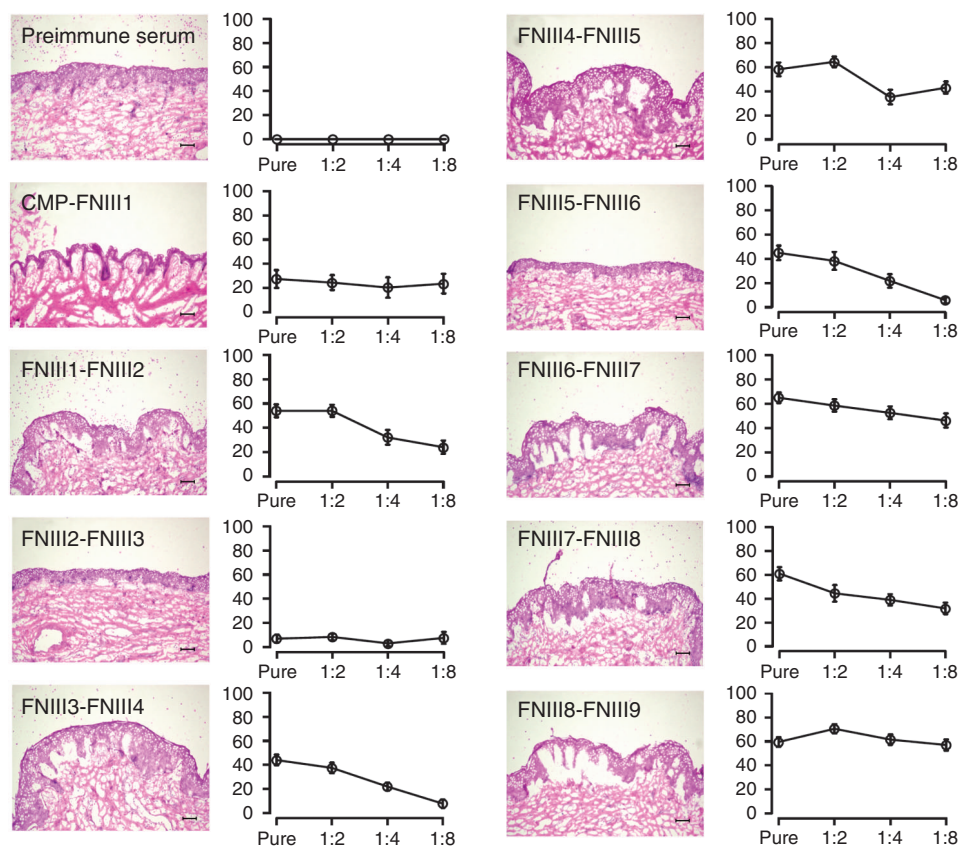
Figure 4. Anti-type VII collagen (anti-COL7) non-collagenous 1 (NC1) antibodies induces dermal–epidermal separation *ex vivo* independent of the targeted epitope. Cryosections of human skin were incubated with epitope-specific rabbit anti-human COL7 NC1 antibodies for 1 hour and subsequently with leukocytes from healthy volunteers for 3 hours. All sera were used as pure 1:2, 1:4, and 1:8 dilutions. On the *y* axis, the percentage of dermal–epidermal separation is shown. Each experiment was performed five times with leukocytes from five volunteers. SEM is depicted. Representative results for each experiment are shown (bar = 100 μm).

generated immune complexes using the different affinity purified antibodies and recombinant NC1 protein. In parallel to above findings using rabbit immune sera, immune complexes of NC1 and either one of the affinity-purified IgG were able to activate neutrophils (Supplementary Figure S2C online).

Antibodies to different epitopes of COL7 cause blister formation when injected into COL7$^{m-/-,h}$ mice

To validate findings from the *ex vivo* assay *in vivo*, affinity-purified antibodies against FNIII4-FNIII5 and FNIII7-FNIII8 subdomains were injected into COL7$^{m-/-,h+}$ (COL7-humanized) mice. Injection of either one of these antibodies into COL7-humanized mice induced clinical and histological changes resembling human EBA (Figure 5). In detail, skin lesions were apparent already on the fourth day after initial injection of antibodies. To assess whether the effect of these anti-COL7 antibodies was dose dependent, lower doses were injected. In fact, these experiments showed a dose-dependent effect of the antibodies in the skin of COL7-humanized mice. To further characterize this EBA mouse model, F(ab)2 fragments of these anti-COL7 IgG were generated. When injected at an equimolar concentration, mixture of anti-FNIII4-FNIII5 and anti-FNIII7-FNIII8 induced subepidermal blisters, the corresponding F(ab)2 fragments did not induce

any clinical or microscopic phenotype (Supplementary Figure S4 online). Investigation of the infiltrating cells showed a predominance of neutrophils in the skin lesions (Figure 5, Supplementary Figure S4 online).

DISCUSSION

We here performed a detailed epitope mapping of reactivity to COL7 using 69 sera from EBA patients. By immunoblotting, we demonstrate a broad spectrum of epitopes located within the NC1 domain that are targeted by the autoantibodies. We next demonstrated that autoantibodies to different epitopes induce blisters *ex vivo*. This finding was confirmed by injection of two different anti-COL7 antibodies into mice carrying null mutations of mouse COL7 and the human COL7 transgene (COL7$^{m-/-,h+}$), which induced subepidermal blisters resembling EBA. Although previous studies clearly demonstrated *in vivo* pathogenicity of autoantibodies to mouse COL7 (Chen *et al.*, 2007; Sitaru *et al.*, 2005; Woodley *et al.*, 2005; Woodley *et al.*, 2006), we here provide *in vivo* evidence of the pathogenicity of autoantibodies directed to human COL7.

According to clinical presentation, mechanobullous and inflammatory EBA is distinguished. In addition, clinical presentation of an individual EBA patient may change during the course of the disease or may show two different pre-
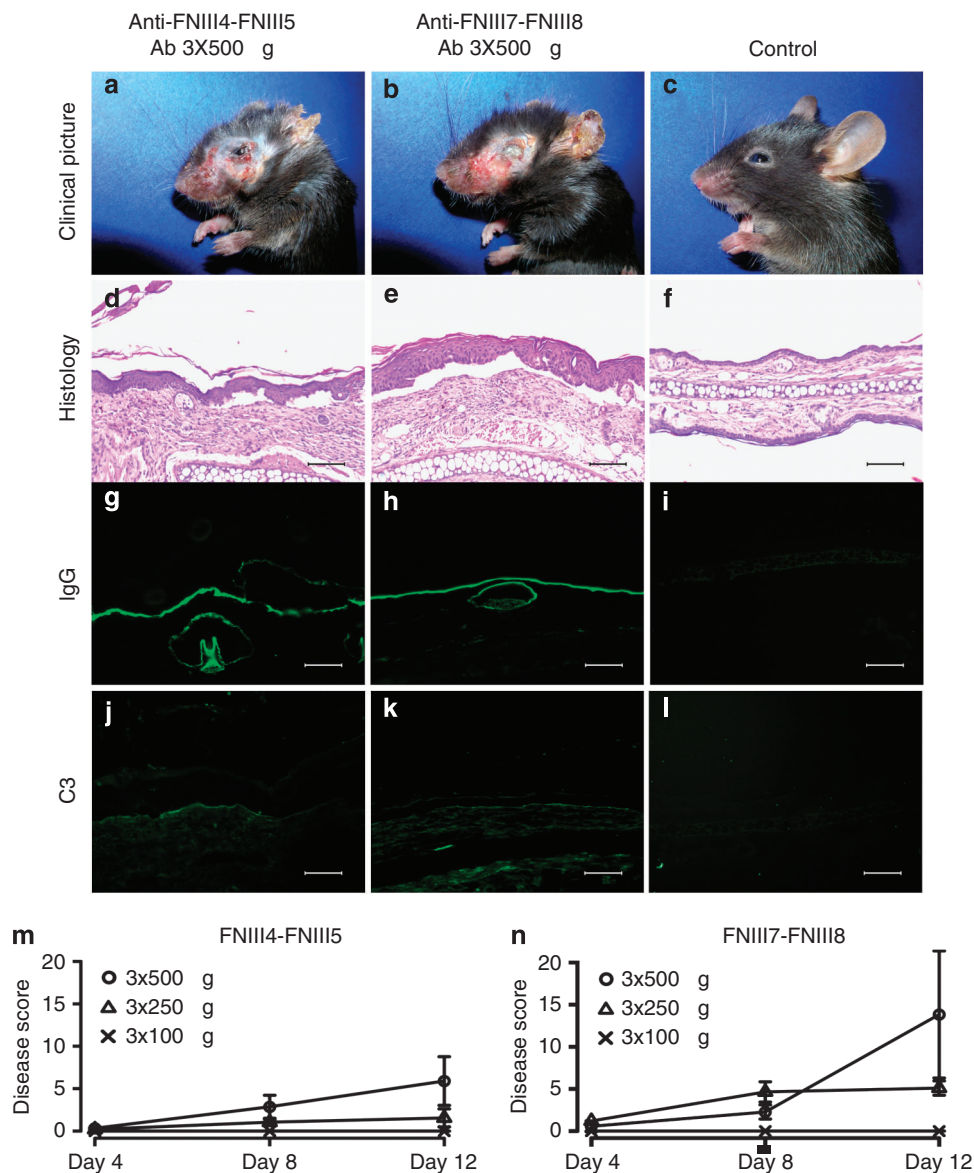
Figure 5. Transfer of anti-FNIII4-FNIII5 or anti-FNIII7-FNIII8 antibodies induces blisters in type VII collagen (COL7)-humanized mice. COL7-humanized mice, injected with $3 \times 500\,\mu g$ of (**a, d**) FNIII4-FNIII5- or (**b, e**) FNIII7-FNIII8-specific antibodies, developed blisters and erosions clinically and subepidermal splits histologically, whereas (**c, f**) control mice remained healthy (bar = 100 µm). By direct immunofluorescence microscopy, COL7-humanized mice injected with FNIII4-FNIII5- or FNIII7-FNIII8-specific antibodies showed (**g, h**) strong IgG and (**j, k**) weak C3 deposits, respectively, at the dermal–epidermal junction, whereas no immunoreactants were found in (**i, l**) control mice (bar = 25 µm). Graphs show disease scores corresponding to body surface area affected by skin lesions after injecting COL7-humanized mice with $3 \times 500\,\mu g$, $3 \times 250\,\mu g$, or $3 \times 100\,\mu g$ of (**m**) anti-FNIII4-FNIII5, or (**n**) anti-FNIII7-FNIII8 affinity-purified antibodies. SDs are indicated.

sentations simultaneously. The clinical distinction between mechanobullous and inflammatory EBA has so far, however, not been reflected by laboratory findings—e.g., autoantibody subclasses (Gupta *et al.*, 2012). As binding of autoantibodies to the vWFA2-domain of NC1 leads to disruption of COL7/ COL1 interactions, as shown by nuclear magnetic resonance (Leineweber *et al.*, 2011), and as interactions of COL7 with both COL4 and laminin-332 have been described (Brittingham *et al.*, 2006; Chen *et al.*, 1997; Rousselle *et al.*, 1997), we hypothesized that autoantibody binding to certain epitopes within the NC1 domain of COL7 is associated with a distinct EBA phenotype. However, in our cohort of 69 EBA patients, we found no correlation of clinical phenotype with

the fine specificity of anti-COL7 IgG. We do not think that reactivity to the collagenous domain of COL7 can explain these findings, as only very few patients had been described to have reactivity to epitopes located within the collagenous domain and/or the hinge region of COL7 (Ishii *et al.*, 2009). Hence, other factors are likely to cause the different clinical phenotypes. In an antibody-transfer model of the disease, inbred mouse lines showed different susceptibilities to blister induction; e.g., in C57BL6/J, the extent of clinical disease was higher compared with BALB/C mice (Kasperkiewicz *et al.*, 2012). As the immune system differs among inbred mouse lines, mechanobullous EBA may develop in patients who do not mount a strong inflammatory reaction in response to

binding of autoantibodies to COL7, if the autoantibody targets a structurally relevant epitope. Careful clinical observations together with stringent immunophenotyping—e.g., analyzing the patients' neutrophils for the ability to mount an oxidative burst upon stimulation with autoimmune complexes—will allow testing this assumption. In line with this assumption, a major interindividual variation of immune complex-induced neutrophil activation has been described (Recke *et al.*, 2010).

After demonstrating a broad epitope recognition of EBA patient IgG, we next evaluated the potential of antibodies targeting different epitopes of NC1 to induce subepidermal splits. When these antibodies were tested for reactivity with the proteins used for immunization, binding to epitopes outside these proteins was observed for anti-FNIII3-FNIII4, anti-FNIII5-FNIII6, anti-FNIII6-FNIII7, anti-FNIII7-FNIII8, and anti-FNIII8-FNIII9 antibodies. This phenomenon may be explained by the high homology among the FNIII-like repeat domains. Hence, in EBA patients, an autoantibody may cross-react with different epitopes on COL7. In addition to inter- and intramolecular epitope spreading (Di Zenzo *et al.*, 2011), this binding of an individual antibody to different epitopes underscores the complexity of the autoimmune response in acquired bullous dermatoses. Although the epitope mapping study used IgG4 as a surrogate marker for the total IgG reactivity to COL7 for sensitivity and specificity purposes, we here either used whole patient IgG or used whole immune IgG from rabbits.

We further showed that all antibodies generated here, which were directed to different epitopes of human COL7 NC1, led to dermal–epidermal separation *ex vivo*, as reported previously using other fragments of COL7 (Csorba *et al.*, 2010). Split formation was dependent on the presence of neutrophils, whereas incubation of human skin with antibodies alone did not cause pathology. This is in line with previous observations demonstrating that patients' antibodies, purified against the entire human NC1 domain, depend on the presence of neutrophils (Sitaru *et al.*, 2002a), more specifically, on generation of reactive oxygen species (Chiriac *et al.*, 2007) and proteases (Shimanovich *et al.*, 2004). To validate the blister-inducing potential of the generated antibodies *in vivo*, rabbit anti-FNIII4-FNIII5 or anti-FNIII7-FNIII8 was injected into COL7-humanized mice. Both these antibody preparations bound to the dermal–epidermal junction, led to complement deposition, and induced subepidermal blisters in a dose-dependent manner, resembling the inflammatory variant of human EBA. This blistering phenotype underscores the pathogenic relevance of the used antibodies, as mere binding of autoantibodies to the dermal–epidermal junction, i.e. formation of immune complexes, does not inevitably lead to blister formation. For example, this is reflected by the lack of blister formation, despite of IgG deposition at the dermal–epidermal junction in mouse models of EBA (Ludwig *et al.*, 2012; Sitaru *et al.*, 2006) and bullous pemphigoid (Liu *et al.*, 1995). This EBA animal model also provides direct evidence that binding of antibodies to human COL7 induces blistering *in vivo*. Previous mouse models of EBA demonstrated pathogenicity of antibodies, directed to mouse or human COL7, that bound murine COL7 *in vivo* (Chen *et al.*, 2007; Sitaru *et al.*, 2006; Sitaru *et al.*, 2005;

Woodley *et al.*, 2005). The pathogenicity of different IgG subclasses, as well as IgA directed against COL7, still remains a controversial issue: although some data suggest a potential of IgG4 to induce subepidermal splits *ex vivo* (Mihai *et al.*, 2007), others have demonstrated opposite findings (Recke *et al.*, 2010). Furthermore, IgA has recently been demonstrated to lead to subepidermal splits *ex vivo* (Recke *et al.*, 2014; van der Steen *et al.*, 2012). On the basis of the developed animal model here, these findings can now be addressed *in vivo* to investigate the potential pathogenic contribution of IgA, as well as IgG subclasses in EBA.

In summary, our mapping studies with NC1 and NC2 domains of COL7 show that autoantibodies in EBA patients recognize a broad spectrum of epitopes within COL7 NC1. No correlation was detected between antibody specificity and clinical phenotype. Both *ex vivo* and using COL7 humanized mouse *in vivo*, these autoantibodies induce subepidermal blisters, independent of their specificity. Furthermore, we demonstrate intramolecular cross-reactivity of autoantibodies directed to a specific epitope within the NC1 domain.

MATERIALS AND METHODS

A more detailed description of the materials and methods used is provided in the Supplementary Materials and Methods online.

Patient sera and human skin

Serum samples from 69 EBA patients for detection of circulating anti-COL7 antibodies were used in this study. As the presence of circulating anti-COL7 antibodies was required for epitope mapping studies, EBA patients diagnosed by detection of an u-serrated pattern in the absence of circulating autoantibodies (Buijsrogge *et al.*, 2011) were not included. Sera from healthy volunteers served as controls. Neonatal human foreskin, obtained from routine circumcision, was snap, cut in 6 μm sections, and mounted on SuperFrost slides (Thermo Scientific, Braunschweig, Germany). Prior to all procedures, written informed consent was obtained from all patients. The study was approved by the ethics committee of the University of Lübeck and was performed according to the Declaration of Helsinki.

Mice

COL7$^{m-/-,h+}$ (COL7-humanized) mice that carry the homozygous null mutations of mouse Col7a1 genes and the transgene of human COL7A1 were generated as described previously (Ito *et al.*, 2009). COL7-humanized mice aged 6–8 weeks were intraperitoneally injected with 100–500 μg in a total of rabbit antibody specific to NC1 domain of human COL7 or phosphate-buffered saline (PBS) at days 0, 2, and 4. The extent of blistering was evaluated at days 4, 8, and 12 after the initial IgG injection. Skin samples for hematoxylin and eosin staining and direct immunofluorescence microscopy and serum samples were obtained at day 12. All animal procedures were conducted according to guidelines of the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol.

Cloning of cDNA clones for human NC1 and NC2 domains of COL7 recombinant proteins

DNA sequence of the NC1 and NC2 domains was obtained from Uniprot database (www.uniprot.org).

Expression and purification of recombinant proteins

E. coli Rosetta DE3 cells were transfected with plasmids. Recombinant proteins were purified by affinity chromatography using chitin beads (New England Biolabs, Ipswich, MA). As proteins CMP-FNIII1 and FNIII9-vWFA2 were purified from inclusion bodies as described (Hirose *et al.*, 2011), they were refolded using oxidized-reduced glutathione buffer, dialyzed against protein elution buffer, and purified by affinity chromatography over chitin beads.

Western blotting with patient sera

Please see Supplementary Materials and Methods online.

Rabbit polyclonal antibody production

Rabbit polyclonal antibodies against different fragments of COL7 were produced as previously described (Sitaru *et al.*, 2005). IgG was purified from preimmune and postimmune serum using protein G-agarose as described (Sitaru *et al.*, 2005). Corresponding F(ab)2 fragments were generated using the F(ab)2 preparation kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol.

Indirect immunofluorescence microscopy

Indirect immunofluorescence studies were performed on murine and 1 M NaCl-split human skin. Cryosections of mouse skin and human salt-split skin of 6-μm thickness were incubated with serial dilutions of immune rabbit serum for 1 hour at room temperature, washed with PBS, and then treated with 1:100 diluted FITC-conjugated polyclonal swine-anti rabbit Ig (Dako, Hamburg, Germany).

Affinity purification of antibodies from rabbit serum

Recombinant proteins were dialyzed in 0.1 M MOPS, pH 7.5, and coupled to Affi-Gel 10 or 15 (according to isoelectric point of proteins) following the manufacturer's protocol (Bio-Rad, Munich, Germany). IgG previously purified from postimmune serum was affinity-purified using corresponding Affi-Gel coupled recombinant proteins, then dialyzed against PBS, and concentrated by Amicon Ultra (30 KDa, Millipore, Billerica, MA).

Affinity purification of antibodies from EBA patient serum

Plasmapheresis material from EBA patient was affinity-purified using corresponding Affi-Gel coupled recombinant proteins, then dialyzed against PBS, and concentrated by Amicon Ultra (30 KDa, Millipore, Billerica, MA).

Purification of normal human leukocytes

Leukocytes were isolated from heparin-anticoagulated blood of five different healthy volunteers following a published protocol (Sitaru *et al.*, 2002b). Leukocytes used in this study (determined by trypan blue) had a viability of ≥90%.

Immune-complex activation of human leukocytes *in vitro*

Please see Supplementary Materials and Methods online.

Determination of dermal–epidermal separation *ex vivo*

Cryosections of 6-μm thickness human foreskin were incubated with different dilutions of rabbit serum (pure, 1:2, 1:4, 1:8, 1:16) for 1 hour at 37 °C, washed with PBS, and subsequently incubated with normal human leukocytes from five different healthy donors at 37 °C for 3 hours. As a negative control, preimmune rabbit serum was used. Subsequently, sections were washed and stained with hematoxylin and eosin. Percentage of dermal–epidermal separation was evaluated by two investigators unaware of the section treatment.

Statistical analysis

R was used for statistical analyses (R Foundation for Statistical Computing, Vienna, Austria). A *P*-value <0.05 was considered significant. Correlation of epitopes with gender, age, and clinical phenotype was assessed by homogeneity distribution analysis and Pearson's correlation. For Pearson's correlation, the coefficient analysis WGCNA package was used. Homogeneity distribution analysis was performed using homals package.

SUPPLEMENTARY MATERIAL

Supplementary Material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Bach JF (2002) The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 347:911–20

Bernard P, Prost C, Aucouturier P *et al.* (1991) The subclass distribution of IgG autoantibodies in cicatricial pemphigoid and epidermolysis bullosa acquisita. *J Invest Dermatol* 97:259–63

Brittingham R, Uitto J, Fertala A (2006) High-affinity binding of the NC1 domain of collagen VII to laminin 5 and collagen IV. *Biochem Biophys Res Commun* 343:692–9

Buijsrogge JJ, Diercks GF, Pas HH *et al.* (2011) The many faces of epidermolysis bullosa acquisita after serration pattern analysis by direct immunofluorescence microscopy. *Br J Dermatol* 165:92–8

Chen M, Doostan A, Bandyopadhyay P *et al.* (2007) The cartilage matrix protein subdomain of type VII collagen is pathogenic for epidermolysis bullosa acquisita. *Am J Pathol* 170:2009–18

Chen M, Marinkovich MP, Veis A *et al.* (1997) Interactions of the amino-terminal noncollagenous (NC1) domain of type VII collagen with extracellular matrix components. A potential role in epidermal-dermal adherence in human skin. *J Biol Chem* 272:14516–22

Chiriac MT, Roesler J, Sindrilaru A *et al.* (2007) NADPH oxidase is required for neutrophil-dependent autoantibody-induced tissue damage. *J Pathol* 212:56–65

Csorba K, Sesarman A, Oswald E *et al.* (2010) Cross-reactivity of autoantibodies from patients with epidermolysis bullosa acquisita with murine collagen VII. *Cell Mol Life Sci* 67:1343–51

Dahl MG (1979) Epidermolysis bullosa acquisita–a sign of cicatricial pemphigoid? *Br J Dermatol* 101:475–84

Di Zenzo G, Thoma-Uszynski S, Calabresi V *et al.* (2011) Demonstration of epitope-spreading phenomena in bullous pemphigoid: results of a prospective multicenter study. *J Invest Dermatol* 131:2271–80

Fine JD, Tyring S, Gammon WR (1989) The presence of intra-lamina lucida blister formation in epidermolysis bullosa acquisita: possible role of leukocytes. *J Invest Dermatol* 92:27–32

Gammon WR, Briggaman RA, Woodley DT *et al.* (1984) Epidermolysis bullosa acquisita–a pemphigoid-like disease. *J Am Acad Dermatol* 11:820–32

Gammon WR, Murrell DF, Jenison MW *et al.* (1993) Autoantibodies to type VII collagen recognize epitopes in a fibronectin-like region of the noncollagenous (NC1) domain. *J Invest Dermatol* 100:618–22

Gandhi K, Chen M, Aasi S *et al.* (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* 20:416–23

Gupta R, Woodley DT, Chen M (2012) Epidermolysis bullosa acquisita. *Clin Dermatol* 30:60–9

Hirose M, Recke A, Beckmann T *et al.* (2011) Repetitive immunization breaks tolerance to type XVII collagen and leads to bullous pemphigoid in mice. *J Immunol* 187:1176–83

Ishii N, Hamada T, Dainichi T *et al.* (2010) Epidermolysis bullosa acquisita: what's new? *J Dermatol* 37:220–30

Ishii N, Yoshida M, Hisamatsu Y *et al.* (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* 150:843–51

Ishii N, Yoshida M, Ishida-Yamamoto A *et al.* (2009) Some epidermolysis bullosa acquisita sera react with epitopes within the triple-helical collagenous domain as indicated by immunoelectron microscopy. *Br J Dermatol* 160:1090–3

Ito K, Sawamura D, Goto M *et al.* (2009) Keratinocyte-/fibroblast-targeted rescue of Col7a1-disrupted mice and generation of an exact dystrophic epidermolysis bullosa model using a human COL7A1 mutation. *Am J Pathol* 175:2508–17

Iwata H, Bieber K, Tiburzy B *et al.* (2013) B cells, dendritic cells, and macrophages are required to induce an autoreactive CD4 helper T cell response in experimental epidermolysis bullosa acquisita. *J Immunol* 191:2978–88

Jones DA, Hunt SW 3rd, Prisayanh PS *et al.* (1995) Immunodominant autoepitopes of type VII collagen are short, paired peptide sequences within the fibronectin type III homology region of the noncollagenous (NC1) domain. *J Invest Dermatol* 104:231–5

Kasperkiewicz M, Nimmerjahn F, Wende S *et al.* (2012) Genetic identification and functional validation of FcgammaRIV as key molecule in autoantibody-induced tissue injury. *J Pathol* 228:8–19

Kim JH, Kim YH, Kim SC (2011) Epidermolysis bullosa acquisita: a retrospective clinical analysis of 30 cases. *Acta Derm Venereol* 91:307–12

Komorowski L, Muller R, Vorobyev A *et al.* (2013) Sensitive and specific assays for routine serological diagnosis of epidermolysis bullosa acquisita. *J Am Acad Dermatol* 68:e89–95

Kurzhals G, Stolz W, Meurer M *et al.* (1991) Acquired epidermolysis bullosa with the clinical feature of Brunsting-Perry cicatricial bullous pemphigoid. *Arch Dermatol* 127:391–5

Lapiere JC, Woodley DT, Parente MG *et al.* (1993) Epitope mapping of type VII collagen. Identification of discrete peptide sequences recognized by sera from patients with acquired epidermolysis bullosa. *J Clin Invest* 92:1831–9

Leineweber S, Schonig S, Seeger K (2011) Insight into interactions of the von-Willebrand-factor-A-like domain 2 with the FNIII-like domain 9 of collagen VII by NMR and SPR. *FEBS Lett* 585:1748–52

Liu Z, Diaz LA, Swartz SJ *et al.* (1995) Molecular mapping of a pathogenically relevant BP180 epitope associated with experimentally induced murine bullous pemphigoid. *J Immunol* 155:5449–54

Ludwig RJ (2012) Model systems duplicating epidermolysis bullosa acquisita: a methodological review. *Autoimmunity* 45:102–10

Ludwig RJ (2013) Clinical presentation, pathogenesis, diagnosis, and treatment of epidermolysis bullosa acquisita. *ISRN Dermatology* 2013:25

Ludwig RJ, Kalies K, Kohl J *et al.* (2013) Emerging treatments for pemphigoid diseases. *Trends Mol Med* 19:501–12

Ludwig RJ, Muller S, Marques A *et al.* (2012) Identification of quantitative trait loci in experimental epidermolysis bullosa acquisita. *J Invest Dermatol* 132:1409–15

Mihai S, Chiriac MT, Herrero-Gonzalez JE *et al.* (2007) IgG4 autoantibodies induce dermal-epidermal separation. *J Cell Mol Med* 11:1117–28

Oostingh GJ, Sitaru C, Zillikens D *et al.* (2005) Subclass distribution of type VII collagen-specific autoantibodies in patients with inflammatory bowel disease. *J Dermatol Sci* 37:182–4

Parente MG, Chung LC, Ryynanen J *et al.* (1991) Human type VII collagen: cDNA cloning and chromosomal mapping of the gene. *Proc Natl Acad Sci U S A* 88:6931–5

Recke A, Sitaru C, Vidarsson G *et al.* (2010) Pathogenicity of IgG subclass autoantibodies to type VII collagen: induction of dermal-epidermal separation. *J Autoimmun* 34:435–44

Recke A, Trog LM, Pas HH *et al.* (2014) Recombinant human IgA1 and IgA2 autoantibodies to type VII collagen induce subepidermal blistering ex vivo. *J Immunol* 193:1600–8

Roenigk HH Jr., Ryan JG, Bergfeld WF (1971) Epidermolysis bullosa acquisita. Report of three cases and review of all published cases. *Arch Dermatol* 103:1–10

Rose NR, Bona C (1993) Defining criteria for autoimmune diseases (Witebsky's postulates revisited). *Immunol Today* 14:426–30

Rousselle P, Keene DR, Ruggiero F *et al.* (1997) Laminin 5 binds the NC-1 domain of type VII collagen. *J Cell Biol* 138:719–28

Sakai LY, Keene DR, Morris NP *et al.* (1986) Type VII collagen is a major structural component of anchoring fibrils. *J Cell Biol* 103:1577–86

Saleh MA, Ishii K, Kim YJ *et al.* (2011) 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* 62: 169–75

Schmidt E, Zillikens D (2013) Pemphigoid diseases. *Lancet* 381:320–32

Shimanovich I, Mihai S, Oostingh GJ *et al.* (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* 204:519–27

Sitaru C, Chiriac MT, Mihai S *et al.* (2006) Induction of complement-fixing autoantibodies against type VII collagen results in subepidermal blistering in mice. *J Immunol* 177:3461–8

Sitaru C, Kromminga A, Hashimoto T *et al.* (2002a) Autoantibodies to type VII collagen mediate Fcgamma-dependent neutrophil activation and induce dermal-epidermal separation in cryosections of human skin. *Am J Pathol* 161:301–11

Sitaru C, Mihai S, Otto C *et al.* (2005) Induction of dermal-epidermal separation in mice by passive transfer of antibodies specific to type VII collagen. *J Clin Invest* 115:870–8

Sitaru C, Schmidt E, Petermann S *et al.* (2002b) Autoantibodies to bullous pemphigoid antigen 180 induce dermal-epidermal separation in cryosections of human skin. *J Invest Dermatol* 118:664–71

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* 102:706–9

van der Steen LP, Bakema JE, Sesarman A *et al.* (2012) Blocking Fcalpha receptor I on granulocytes prevents tissue damage induced by IgA autoantibodies. *J Immunol* 189:1594–601

Woodley DT, Briggaman RA, O'Keefe EJ *et al.* (1984) Identification of the skin basement-membrane autoantigen in epidermolysis bullosa acquisita. *N Engl J Med* 310:1007–13

Woodley DT, Chang C, Saadat P *et al.* (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* 124:958–64

Woodley DT, O'Keefe EJ, Reese MJ *et al.* (1986) Epidermolysis bullosa acquisita antigen, a new major component of cutaneous basement membrane, is a glycoprotein with collagenous domains. *J Invest Dermatol* 86:668–72

Woodley DT, Ram R, Doostan A *et al.* (2006) Induction of epidermolysis bullosa acquisita in mice by passive transfer of autoantibodies from patients. *J Invest Dermatol* 126:1323–30

Zambruno G, Manca V, Kanitakis J *et al.* (1994) Linear IgA bullous dermatosis with autoantibodies to a 290 kd antigen of anchoring fibrils. *J Am Acad Dermatol* 31:884–8