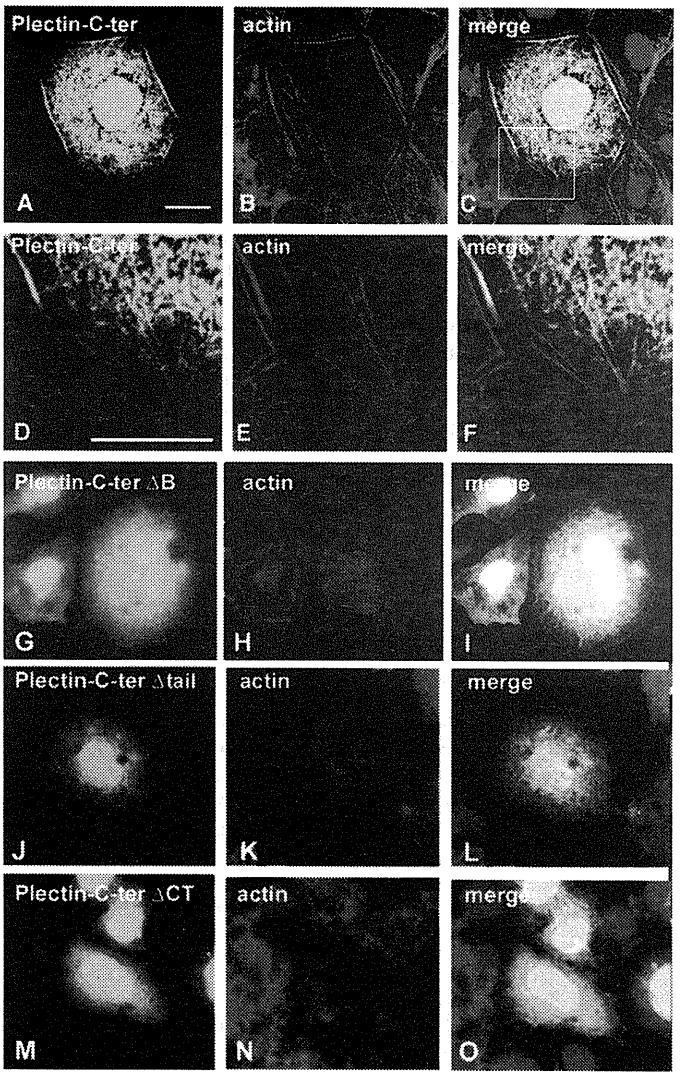

Fig. 7. Immunofluorescent staining of Plectin-C-ter, its short fragments and actin microfilaments in SW-13 VIM−/− cells. A, D, Plectin-C-ter; B, E, actin microfilaments; C, F, merged images. (A–C) Low magnification image; (D–F), higher magnification image of box area indicated in C. Note the identical expression of the Plectin-C-ter and actin filaments in some parts. (G–I) G, Plectin-C-ter ΔB repeat; H, actin microfilaments; I, merged images. (J–L) J, Plectin-C-ter Δtail; K, actin microfilaments; L, merged images. (M–O) M, Plectin-C-ter ΔCT; N, actin microfilaments; O, merged images. The scale bar represents 20 μm.

structures on the surface of the C-terminal globular domain of plectin that can bind to other proteins [6]. Fontao et al. reported similar roles of the linker regions for other members of the plakin family, namely, desmoplakins and bullous pemphigoid antigen-1e (BPAG1e) [19].

Intriguingly, the transfection of plectin-C-ter ΔCT, Δtail and ΔBC repeat construct caused at least partial inhibition of plectin to IF binding in COS7 cells and NHKs. We do not know the discrepancy between previous 4 reports and ours in light of the role of the binding of plectin C repeat and tail to IFs. However, it may be due to the difference of the experimental design.

There is increasing evidence to indicate that plectin–IF interactions are regulated by protein kinase-dependent phosphorylation. For example, the interaction between plectin and lamin B is significantly decreased by phosphorylation of plectin by cAMP-dependent protein kinase (PKA) or protein kinase C (PKC). By contrast, binding between plectin and vimentin is increased after PKA-phosphorylation but is decreased after PKC-phosphorylation [21]. We also found here that the serine residue at position 4645 in the tail domain of the C-terminus may have a role in the binding of plectin to vimentin IFs. Currently, the candidate protein kinase of this residue is not known. In desmoplakin, the corresponding serine residue in the COOH-terminal domain is believed to be phosphorylated in a protein kinase A-dependent manner [19]. Interestingly, although the corresponded mutation in desmoplakin caused stronger binding of desmoplakin to IF [22], the same mutation in plectin reported here caused weaker binding of plectin to IF at the periphery of COS7 cells but normal binding of the other portion of cells. Moreover, aggregated co-localization of plectin and K14 was observed in NHKs. We speculated that phosphorylation of this site plays some roles of plectin–IF binding. The phosphorylation of this site may stabilize the binding of plectin to IF at the cell periphery and may regulate the binding of plectin to IF in the other cell area. Further study is required to elucidate this.

Other intriguing issue to be discussed is that in vimentin-null SW-13 VIM−/− cells transfected with plectin-C-ter mutants, all the mutant proteins did not co-localize with and possibly disrupted actin microfilaments. The ability of each plectin-C-ter mutant to disrupt actin microfilaments did not correspond with that of each construct to IFs. As these results were still preliminary, further study is required to fully elucidate this.

In conclusion, we have shown that the linker region of the plectin C-terminus mediates the association of plectin with IFs. We also have demonstrated that the plectin C-terminus tail fragment may also play some roles on the association of plectin with IFs.

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ORIGINAL ARTICLE

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 10.1016/j.jaad.2011.12.032.)

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

From the Institute of Experimental Immunology, EUROIMMUN AG,[a] Department of Dermatology,[b] and Comprehensive Center for Inflammation Medicine,[g] University of Luebeck; Department of Dermatology, University Medical Center Groningen, University of Groningen[c]; Department of Dermatology, Kurume University School of Medicine and Kurume University Institute of Cutaneous Cell Biology[d]; Department of Dermatology and Cutaneous Biology Research Institute, Yonsei University College of Medicine, Gangnam Severance Hospital,[e] Seoul; and the Department of Immunodermatology, St John's Institute of Dermatology, St Thomas' Hospital,[f] London.

Drs Komorowski and Müller contributed equally and are listed in alphabetical order.

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Correspondence to: Enno Schmidt, MD, PhD, Department of Dermatology, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany. E-mail: enno.schmidt@uk-sh.de.

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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]

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.

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 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 (ColVII-

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

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

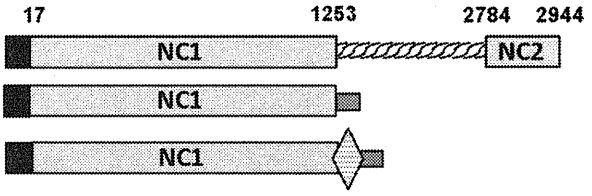

Fig 1. Schematic diagram of type VII collagen (Col VII, *top*) and the HEK293 cell-expressed recombinant non collagenous domain-1 (NC1) of Col VII used for ELISA (*middle*) and immunofluorescence microscopy (*bottom*). Col VII is a homotrimer consisting of an amino-terminal signal sequence (*black box*), 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.

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

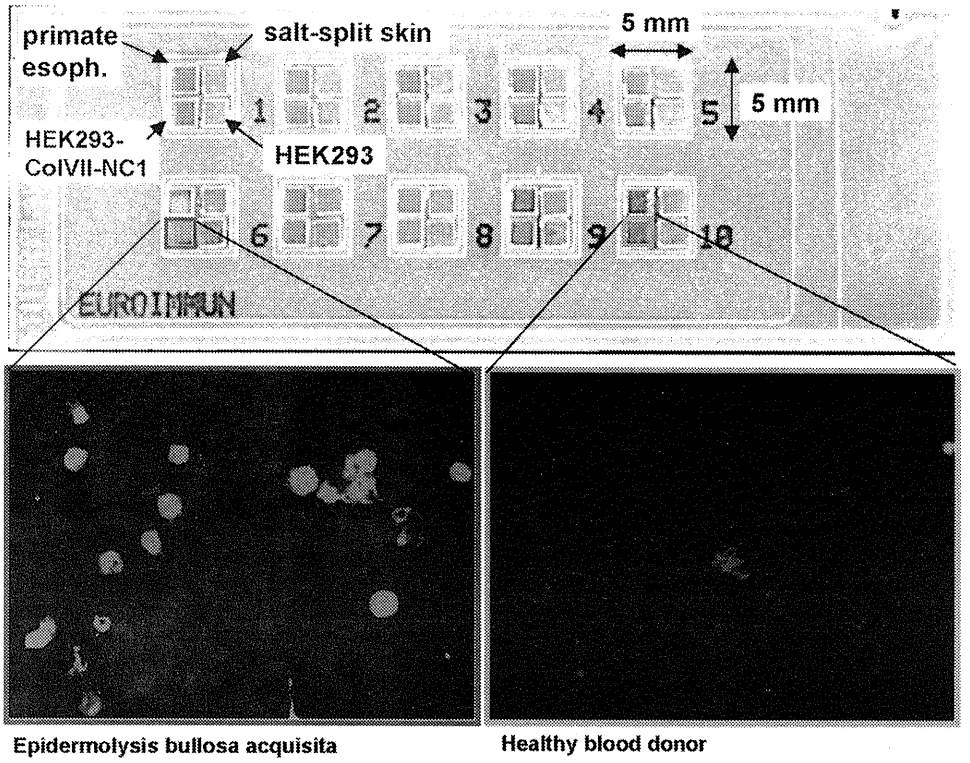

Fig 2. Slide with 10 reaction fields containing HEK293 cells expressing non-collagenous domain-1 of type VII collagen on cell surface via the transmembranous domain of desmoglein 1 (HEK293-ColVII-NC1) and untransfected control cells (HEK293). In the reaction field, additional substrates may be included 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.

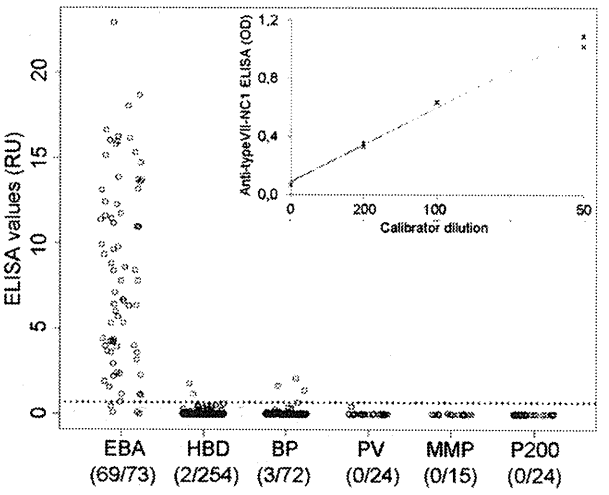

Fig 3. ELISA reactivities of sera from patients with EBA and controls. IgG reactivity to the noncollagenous domain-1 of type VII collagen was found in sera from 69 of 73 (94.5%) EBA patients, 2 of 254 (0.8%) healthy blood donors (HBD), 3 of 72 (4.1%) bullous pemphigoid (BP) patients and none of 24, 15, 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.

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 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,

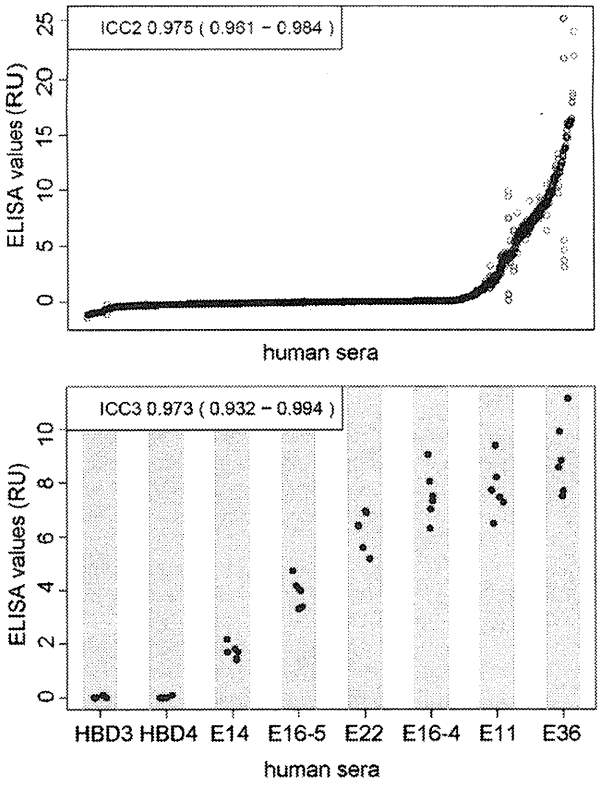

Fig 4. Reproducibility of ELISA measurements. The intra-assay variation was determined by quadruplicate measurements on the same plate with sera (n = 55) covering a broad range of ELISA reactivities. The intraclass coefficient for randomly selected measurements (ICC2) was 0.975 (95% confidence interval [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.

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