transfer of pathogenic autoantibodies [8]. IgG autoantibodies from EBA patients induced dermal-epidermal separation in frozen sections of normal human skin when co-incubated with granulocytes from healthy donors [9]. Further *in vivo* work showed that the passive transfer of collagen VII-specific antibodies into mice induced subepidermal blisters [10]. Immunization with autologous collagen VII induces a T cell-dependent autoimmune response and subepidermal blisters in mice [11-13].

Collagen VII, the main structural component of the anchoring fibrils, is a 290 kDa protein composed of three identical α chains, each consisting of a central collagenase sensitive triple helical portion flanked by a 145 kDa N-terminal (NC1) and a 34 kDa C-terminal (NC2) non-collagenous domains [14,15]. Two molecules of collagen VII associate through a small overlap of the C-terminal NC2 domain resulting in the dimer form present in anchoring fibrils. In the extracellular space, large part of the NC2 domain is proteolytically removed by bone morphogenetic protein 1 (BMP-1), an enzyme with procollagen C-proteinase activity. In spite of this proteolytic cleavage a small peptide of NC2 domain consisting of 41 aminoacids still reside in the dermis, below the lamina densa [16-18]. Epitope mapping studies revealed that the major epitopes recognized by EBA autoantibodies reside within the NC1 domain of native collagen VII [19,20]. In addition to very few cases showing reactivity to the triple helical domain of collagen VII, further important epitopes of EBA autoantibodies have been more recently mapped to the NC2 domain [21,22]. The laboratory diagnosis of EBA relies on several laboratory tests, including detection of tissue-bound autoantibodies by direct IF microscopy and demonstration of serum autoantibody binding to the dermal side of the 1 M salt-split skin by indirect IF microscopy. The definitive diagnosis of EBA requires characterization of the molecular specificity of autoantibodies [1]. Autoantibodies against collagen VII are commonly detected by immunoblotting and/or ELISA using recombinant proteins [1]. While collagen VII-specific autoantibodies are present in virtually all EBA patients, their exact prevalence in patients with IBD or other autoimmune bullous diseases is still controversial [23,24] or unknown, respectively.

For detection of collagen VII-specific IgG autoantibodies by ELISA, immunoassays using recombinant forms of the NC1 domain or the full-length molecule have been developed [22,25-27]. Based on B cell epitope *in silico* prediction and wetlab mapping studies, a recombinant form of collagen VII containing both the NC1 and NC2 domains as well as the hinge region would allow a most sensitive detection of anti-collagen VII antibodies in patients. In addition, expression of a lower molecular mass non-collagenous protein should allow for better protein yields compared with the expression of the full-length collagenous molecule. Therefore, in the present study, we have developed an immunoassay for the detection of collagen VII-specific autoantibodies using a chimeric recombinant fusion protein composed of both non collagenous domains and the hinge region of collagen VII. This protein, expressed in stably transfected HEK-293 cells to ensure optimal posttranslational modifications, contains all putative epitopes of collagen VII in equimolar amounts and was utilized to develop a sensitive ELISA for the detection in the same assay of EBA autoantibodies. Using this immunoassay the prevalence of collagen VII-specific autoantibodies and their IgG subclass was characterized in large cohorts of patients with IBD, pemphigus vulgaris (PV) and bullous pemphigoid (BP) as well as healthy donors.

Methods

Human sera

Serum samples were obtained from patients with EBA (n = 50), Crohn's disease (CD; n = 50), ulcerative colitis (UC; n = 50), BP (n = 76), and PV (n = 42) before initiation of treatment and healthy donors (n = 245). EBA and BP patients were characterized by: (a) subepidermal skin blisters, (b) linear IgA or IgG deposits along the dermal-epidermal junction detected by direct IF microscopy, and (c) circulating IgG autoantibodies binding to the epidermal (BP) or dermal (EBA) side of the salt-split skin as revealed by IF microscopy. PV was diagnosed in patients presenting (a) intraepidermal skin blisters and mucosal or mucocutaneous involvement, (b) intercellular IgG deposits within the epidermis detected by direct immunofluorescence (DIF) microscopy, (c) Serum IgG autoantibodies binding to the epithelium of monkey esophagus with an intercellular pattern by IF microscopy, and (d) IgG autoantibodies against desmoglein 3 by ELISA. Sera from patients with a moderate or severe UC or CD were included. The patients were selected within the IBD section of the Medical Clinic I Erlangen, University of Erlangen-Nuremberg, having a confirmed diagnosis for an IBD entity based on the consensus evidence-based clinical, endoscopical and histopathological criteria [28]. The study was approved by the Ethics Committee of the Medical Faculty of the University of Freiburg, Germany (Institutional Board Projects no 318/07, 425/08 and 278/11). We obtained informed consent from patients whose material was used in the study, in adherence to the Helsinki Principles.

Cell culture

Transfected Flp-In HEK 293 T cells (Flp-In™-293, Invitrogen) were cultured in DMEM medium, with phenol red (Lonza) supplemented with 10% FCS, L-glutamine, penicillin, streptomycin (all from Biochrome). When cells reached 70% confluence, complete growth medium was replaced with serum free medium supplemented with 100 μg/ml of vitamin C. After 48 hours, cell culture medium was

collected, cleared by centrifugation at 3200 × g for 7 min at 4°C and 5 mM EDTA and 1 mM PMSF were added. The supernatant was stored at -20°C until used.

Generation of recombinant noncollagenous domains of human collagen VII

cDNA sequences corresponding to the non-collagenous domains of human collagen VII (NC1, NC2) were obtained by polymerase chain reaction (PCR) amplification on 8xHis tagged full length collagen VII sequence (kind gift from A Fritsch), previously cloned into prokaryotic vector pcDNA3.1 Zeo(-), Invitrogen [29]. Primers for PCR were synthesized by Eurofins MWG (Ebersberg, Germany; Table 1). Restriction sites for EcoRI and HindIII were introduced by primers (Table 1). Briefly, pcDNA3.1hcol7 vector containing the full length sequence of human collagen VII was digested with EcoRI and AgeI restriction enzymes. The digested vector containing the sequence spanning aminoacids 1-443 was ligated with the PCR fragment overlapping the restriction site for AgeI within collagen VII sequence resulting in the recombinant vector pcDNA3.1hCol7NC1 containing the entire sequence of NC1 domain of collagen VII spanning the aminoacids 1-1278. The PCR product corresponding to the NC2 fragment was digested with EcoRI and HindIII restriction enzymes and ligated into pcDNA3.1hCol7NC1 vector digested with the same enzyme resulting in the recombinant vector pcDNA3.1hCol7NC1-NC2 with the sequence spanning the aminoacids 1-1278 and 2776-2944. Subsequently, the recombinant fragment (NC1-NC2) was subcloned into the pcDNA5FRT vector with CMV promoter using NheI and HindIII restriction enzymes resulting pcDNA5FRThcol7NC1-NC2 recombinant vector. To obtain the recombinant protein containing NC1, hinge region and NC2 domains of type VII collagen the nucleotide sequence coding the hinge region and NC2 domain, flanked by the restriction sites for EcoRI and Hind III, was synthesized by GenScript in pUC57 vector. Further, the sequence was cut out with EcoRI and HindIII restriction enzymes and ligated into the pcDNA5FRT NC1-NC2 vector digested with the same enzymes resulting pcDNA5FRThcol7NC1-H-NC2 recombinant vector containing the sequence spanning the aminoacids 1-1278, 1940-1979 and 2776-2944. Correct DNA sequences of all vectors were confirmed by direct secquencing. Flp-in Hek293 T host cells were transfected with 5 μg of pcDNA5FRThCol7NC1-NC2/pcDNA5FRThCol7NC1-H-NC2 and 2.5 μg of pOG44 vectors in lipofectamine2000 (Invitrogen). Transfected cells expressing the desired proteins were selected under 200 μg/ml hygromicine (Roth). Proteins were precipitated from the culture medium with 50% ammonium sulphate for 4 h at 4°C and collected by centrifugation at 27000 × g for 45 minutes at 4°C. The proteins were resuspended in cold PBS, dyalised overnight against PBS and purified by metallochelate affinity chromatography using nickel nitrilotriacetic acid coupled with agarose (Ni-NTA, Qiagen, Germany). Purified proteins were separated by SDS PAGE on 8% gels under reducing conditions and transferred on nitrocellulose membrane. Membrane strips were incubated with 1000-fold diluted monoclonal antibody specific for human collagen VII (clone LH 7.2; Chemicon International, Germany) and reactivity was detected with secondary, HRP-conjugated goat anti-mouse IgG antibodies (Abcam).

Table 1 Primer sequences for PCR amplification of col7a1 cDNA fragments

Fragment	Size (bp)	Primer sequences (5'-3')
NC1	2573	FP: GATCCTGGGCCCCACATCCATCCTC
		RP: GATCGAATTCGCCCGGGAGGCCAGGGTCG
NC2	524	FP: GATCGAATTCGGCGAGAAGGGAGAAGCTGC
		RP: GATCAAGCTTTCAGTCCTGGGCAGTACCTGT

FP forward primer, RP reverse primer

Enzyme-linked immunosorbent assays

ELISA was developed and performed using previously established protocols with modification [30,31]. Briefly, 96-well microtiter plates (Greiner Bio-One, Germany) were coated with 500 ng/well of recombinant His-hCVII-NC1-NC2 and an equimolar amount of His-hCVII-NC1-H-NC2 in 0.1 M bicarbonate buffer (pH 9.6), overnight at 4°C. Next day the plates were washed with 0.05% Tween20-PBS (w/v) and blocked 1 h with 1% BSA-PBS (w/v) followed by incubation with 1:100 diluted sera in 1% BSA-0.05% Tween20-PBS (w/v) for 1 h. Bound antibodies were detected by 1 hour incubation with a mixture of 2000-fold diluted biotin conjugated mouse antibodies recognizing the four human IgG subclasses (Invitrogen) and subsequently with horseradish-peroxidase conjugated Streptavidin (Dianova) diluted 1:250. After washing, color reaction was developed by addition of orthophenylene diamine substrate (Dako). Reaction was stopped after 10 minutes with 0.5 M sulphuric acid solution. All steps were carried out at room temperature. The optical density (OD) was read at 492 nm using an automated spectrophotometer (Sirius HT-TRF, MWG). Each serum was tested in triplicate. The cut-off for positivity was validated and optimized by receiver-operating characteristics (ROC) analysis as described below. The accuracy of the assay was expressed as sensitivity = true positive/(true positive + false negative) and specificity = true negative/(true negative + false positive).

SDS-PAGE and immunoblot analysis

Immunoblotting with recombinant proteins was performed as described with minor modification [31]. Briefly, preparations of recombinant His-hCVII-NC1-NC2 and His-hCVII-NC1-H-NC2 proteins were separated by SDS-

PAGE on 8% preparative gels, under reducing conditions, followed by transfer onto nitrocellulose (Whatman/Protran BA85). Membrane strips were incubated with 100-fold diluted EBA and normal human sera. Reactivity was visualized with secondary, HRP-conjugated goat anti-human IgG antibodies (Abcam) and diaminobenzidine (Merck).

Indirect immunofluorescence

Serum IgG autoantibodies were detected by IF following published protocols [31]. Briefly, frozen sections of salt-split healthy human skin were incubated in a first step with serially diluted sera. IgG antibodies bound at the dermal side were visualized with 100-fold diluted, Alexa Fluor488-labelled polyclonal goat anti-human IgG antibody (Invitrogen).

In silico and statistical analysis

Linear and conformational epitopes on collagen VII were analyzed *in silico* using software available at 3 different web servers. BepiPred predicts the location of linear B-cell epitopes using a combination of a hidden Markov model and a propensity scale method protocol http://www.cbs.dtu.dk/services/BepiPred/[32]. CBTOPE predicts conformational B-cell epitope of an antigen from its amino acid sequence (http://www.imtech.res.in/raghava/cbtope/) [33]. The Predictor component of Epitope Toolkit (EpiT; http://ailab.cs.iastate.edu/bcpreds/) was used to predict flexible length linear B-cell epitopes by the FBCPred algorythm [34]. A ROC curve allows for exploring the relationship between the sensitivity and specificity of the ELISA for a variety of different cut-off points, thus allowing the determination of an optimal cut-off point for positivity. Therefore, to determine the cut-off value for the ELISA using recombinant forms of type VII collagen, we performed a ROC analysis by plotting on the X-axis the 1 - specificity (the false positive rate) and on the Y-axis the sensitivity (the true positive rate). Statistical analyses were performed using the GraphPad Prism statistical package (v5; GraphPad Software, San Diego, CA). Statistical significance was calculated using the non-parametric Mann-Whitney-*U* test and correlations were analyzed by the Spearman's rank correlation test; $p < 0.05$ was considered significant.

Results

In silico prediction of B cell epitope

Wet lab epitope mapping studies using different recombinant fragments of collagen VII showed that the epitopes targeted by autoantibodies are localized within the NC1, NC2 and, possibly, the hinge region of the antigen. To further our understanding about the distribution of B cell epitopes on collagen VII, we applied *in silico* analysis of both linear and conformational epitopes using different algorithms. The results revealed that NC1 and NC2 domains as well as the hinge region within the triple helix of collagen VII contain more antigenic sites compared with its collagenous domain (Figure 1, Additional file 1: Table S1).

Generation of the recombinant forms of the autoantigen

The recombinant proteins were expressed in mammalian cells and purified by metallochelate affinity chromatography. When separated by SDS-PAGE, the recombinant collagen VII forms containing the NC1 fused with the NC2 domain (His-hCVII-NC1-NC2) as well as the hinge region (His-hCVII-NC1-H-NC2), migrated consistently with their calculated molecular masses of 153 kDa (Figure 2b, lane 2) and 158 kDa (Figure 2b, lane 3), respectively. A monoclonal antibody specific for the NC1 domain of collagen VII recognized both recombinant forms by immunoblot analysis (Figure 2c, lanes 1 and 2).

Immunoreactivity of recombinant collagen VII with EBA autoantibodies

The immunoreactivity of the newly expressed recombinant chimeric forms of collagen VII was analyzed by immunoblotting using sera from reference EBA patients and healthy donors. Representatives examples are shown in Figure 3. IgG autoantibodies from EBA patients' sera (n = 5) recognized the recombinant forms His-hCVII-NC1-NC2 (Figure 3, lanes 1-3) and His-hCVII-NC1-H-NC2 (Figure 3, lanes 5-7) of collagen VII. Normal human sera (n = 2) did not react with these recombinant forms of collagen VII (Figure 3, lanes 4 and 8).

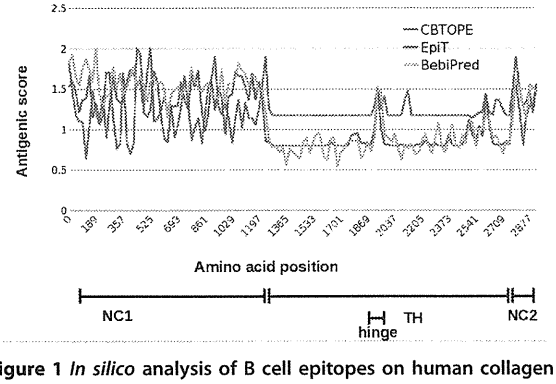

Figure 1 *In silico* analysis of B cell epitopes on human collagen VII. Linear and conformational antigenic determinants of collagen VII were analyzed *in silico* using BepiPred (http://www.cbs.dtu.dk/services/BepiPred/), CBTOPE (http://www.imtech.res.in/raghava/cbtope) and the Predictor component of Epitope Toolkit (EpiT; http://ailab.cs.iastate.edu/bcpreds/). The antigenic scores are plotted for the entire sequence of human collagen VII. The different regions of the autoantigen, including its non-collagenous (NC) 1 and 2 domains as well as the triple helical (TH) and hinge regions are shown in the lower part of the figure.

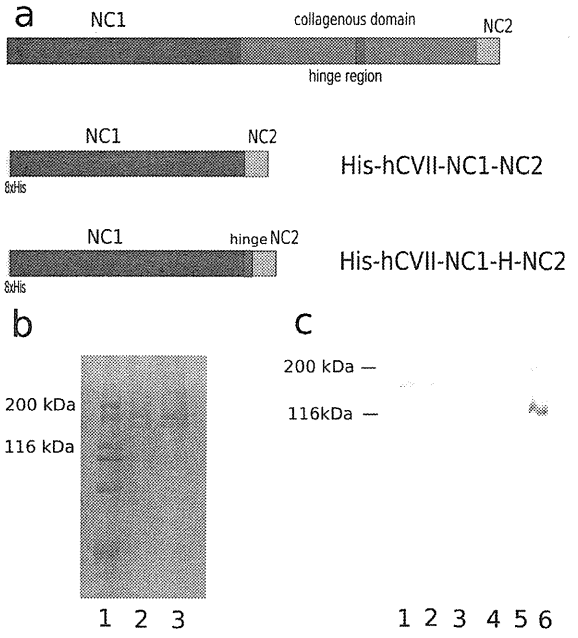

Figure 2 Recombinant forms of collagen VII used in this study. **(a)** Schematic representation of human collagen VII consisting of a central collagenous domain flanked by a large, 145 kDa N-terminal non-collagenous domain and a smaller, 30 kDa non-collagen domain at its C-terminus. The collagenous domain is interrupted by a 39 amino acid non-collagenous hinge region. The recombinant forms of collagen VII generated in this study are two N-terminally 8xhistidine tagged chimeric proteins termed His-hCVII-NC1-NC2 and His-hCVII-NC1-H-NC2 corresponding to the fused NC1 and NC2 domains (aa 1-1278, 2776-2944) and to the fused NC1, hinge and NC2 regions (1-1278, 1940-1979, 2776-2944) of the antigen, respectively. **(b)** Sodium dodecylsulfate-polyacrylamide gel electrophoresis of the purified recombinant His-hCVII-NC1-NC2 and His-hCVII-NC1-H-NC2 proteins shows their migration at around 153 (lane 2) and 158 kDa (lane 3), respectively. Weight markers of 200, 116, 97, 66 and 45 kDa are shown in lane 1. **(c)** Immunoblot analysis of the two recombinant proteins His-hCVII-NC1-NC2 (lanes 1 and 4) and His-hCVII-NC1-H-NC2 (lanes 2 and 5) as well as a recombinant form of collagen XVII [47] (lanes 3 and 6) using a monoclonal antibody specific to the NC1 domain of collagen VII (clone LH7.2; lanes 1-3) and a monoclonal antibody specific to soluble ectodomain of collagen XVII [47] (lanes 4-6).

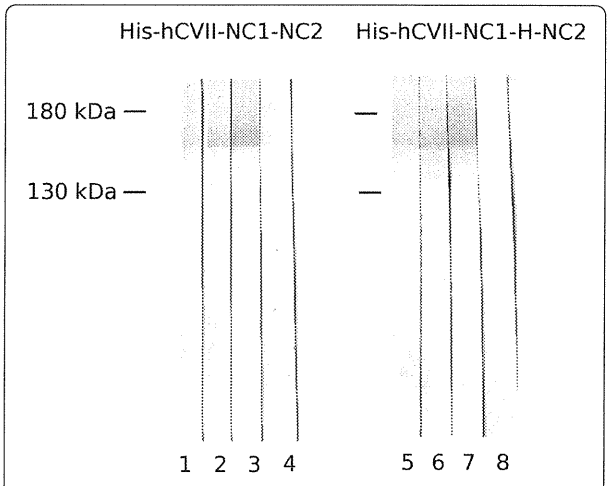

Figure 3 Immunoreactivity of epidermolysis bullosa acquisita (EBA) autoantibodies with the recombinant forms of collagen VII. Purified, recombinant His-hCVII-NC1-NC2 (lanes 1-4) and His-hCVII-NC1-H-NC2 (lanes 5-8) were electrophoretically separated by 8% SDS-PAGE, transferred to nitrocellulose and immunoblotted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

Development of ELISA using recombinant collagen VII

The working conditions, including antigen amount/well, dilution of sera and secondary antibodies have been defined by an initial chessboard titration (data not shown). To determine the cut-off value of the newly established immunoassay, we performed a ROC analysis of the ELISA readings with sera from 50 EBA patients and 160 healthy donors for the ELISA results obtained with both His-hCVII-NC1-NC2 and His-hCVII-NC1-H-NC2. The area under the curve (AUC) was 0.980 (95% CI.: 95%-100%) and 0.984 (95% CI.: 96%-100%) for His-hCVII-NC1-NC2 and His-hCVII-NC1-H-NC2, respectively (Figure 4). Based on a calculated specificity of 97.50% and a sensitivity of 92% (His-hCVII-NC1-NC2) and 94% (His-hCVII-NC1-H-NC2) the cut-off was set at 0.425 and 0.322 OD reading units for His-hCVII-NC1-NC2 and His-hCVII-NC1-H-NC2, respectively.

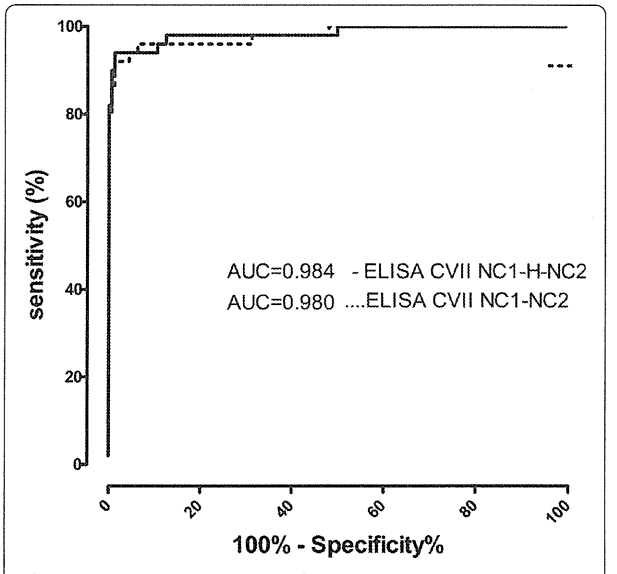

Figure 4 Receiver-operating-characteristic (ROC) curve. AUC, area under the curve. Test performed with sera from patients with epidermolysis bullosa acquisita (n = 50) and controls (n = 160).

ELISA using recombinant NC1-NC2 and NC1-hinge-NC2 forms of collagen VII allows for a sensitive and specific detection of antigen-specific autoantibodies

Applying the cut-off value of 0.322 defined by ROC analysis for the newly developed ELISA showed that 47 EBA (94%; 95% CI: 87%-100%; n = 50), 2 CD (4%; 95% CI: 0%-9.43%; n = 50), 8 UC (16%; 95% CI: 5.8%-26%; n = 50), 2 BP (2.63%; 95% CI: 0%-6.23%; n = 76), 4 PV (9.52%; 95% CI: 0%-18.4%; n = 42) patients and 4 of the healthy donors (1.63%; 95% CI: 0%-3.21%; n = 245) showed IgG reactivity against the chimeric NC1-hinge-NC2-hCVII protein (Figure 5). Therefore, a sensitivity and a specificity of 94% (95% CI: 83.4%-98.75%) and 98%(95% CI: 94%-100%), respectively, were calculated for the ELISA detecting collagen VII-specific IgG autoantibodies in patients with EBA. The area under the curve (AUC) was 0.984 (95% CI: 96.3%-100%) indicating an excellent discriminatory power. The accuracy of the ELISA using only the NC1-NC2 domains of collagen VII was only slightly lower as demonstrated by a sensitivity of 92% (95% CI: 80.7%-97.7%) and a specificity of 97.50% (95% CI: 93.7%-99.3%) with an AUC of 0.980 (data not shown). The immunoassays using the 2 recombinant forms of collagen VII correlated well regarding their capacity to detect specific autoantibodies ($r = 0.95$; $p < 0.0001$).

IgG levels by hCVII ELISA correlate with the IgG reactivity against the dermal-epidermal junction by IF microscopy

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

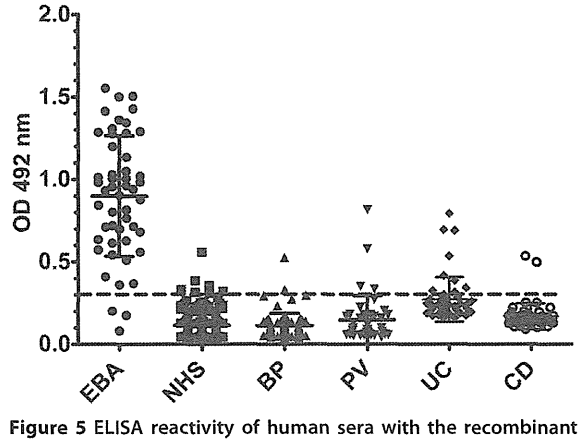

Figure 5 ELISA reactivity of human sera with the recombinant NC1-hinge-NC2 noncollagenous domains of collagen VII. Scatter plots represent optical density measurements of serum reactivity of epidermolysis bullosa acquisita (EBA), bullous pemphigoid (BP), pemphigus vulgaris (PV), Crohn's disease (CD), ulcerative colitis (UC) patients and healthy donors (NHS) with the purified recombinant chimeric collagen VII (His-hCVII-NC1-H-NC2). The cut-off of the assay is represented by a dotted line.

Levels of autoantibodies against collagen VII do not correlate with inflammation markers in inflammatory bowel disease

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

IgG4 autoantibodies dominate the autoimmune response against collagen VII

The IgG subclass of collagen VII-specific autoantibodies in patients' sera was analyzed by ELISA using recombinant His-hCVII-NC1-H-NC2 substrate. In 34%, 37%, 16% and 100% of sera autoantibodies of IgG1, IgG2, IgG3, and IgG4 isotype recognized the recombinant autoantigen (Figure 6). By correlating the results obtained for the IgG subclasses with the values obtained by the standard assay, correlation coefficients of $r = -0.07$ ($p = 0.8$), $r = 0.17$ ($p > 0.1$), $r = 0.24$ ($p > 0.1$) and $r = 0.727$ ($p < 0.001$) resulted for the detection of IgG1, IgG2, IgG3 and IgG4, respectively.

Discussion

Autoimmune phenomena were observed in the development of both cellular and humoral responses. In fact, there may be significant overlap between the autoreactive and protective antibodies since polyreactive antibodies represent a substantial part of the normal repertoire. Antibodies against specific self-antigens are typically associated with systemic or organ-specific autoimmune diseases, but may be also found in neoplastic diseases and even in healthy subjects. EBA is a prototypical organ-specific autoimmune disease affecting the skin and the mucous membranes associated with autoantibodies against collagen VII [1]. The blister-inducing potential of collagen VII-specific

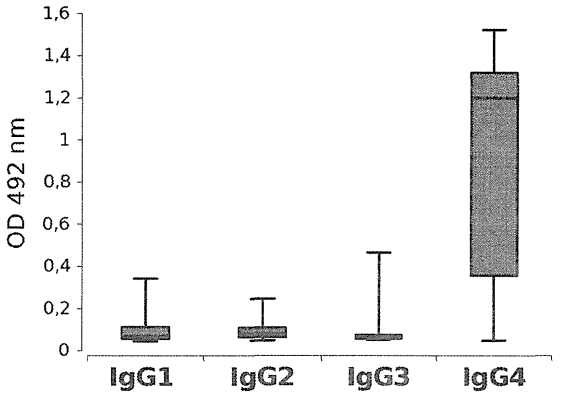

Figure 6 Autoantibodies against collagen VII are mainly of IgG4 isotype. Box-and-whiskers graphs represent descriptive summaries of the measurements for IgG1, IgG2, IgG3 and IgG4 autoantibodies against collagen VII by ELISA as described in Methods.

autoantibodies has been shown in different *ex vivo* and animal models [13]. Autoimmunity against collagen VII has been described in IBD, which may be clinically associated with EBA [2]. Interestingly, collagen VII-specific autoantibodies are present in IBD patients, which do not show blistering skin disease [2]. The aim of the present study, was to analyze collagen VII-specific autoantibodies, including IgG subclasses in large groups of healthy blood donors or patients with autoimmune blistering diseases.

The rapid and accurate routine diagnosis of most autoimmune diseases relies on the detection of autoantibodies with known molecular specificity. Several immunoassays have been developed so far for the detection of autoantibodies against collagen VII (Table 2) [22,25-27]. To further improve the detection of collagen VII-specific autoantibodies, in the present study we have generated a chimeric antigen substrate containing virtually all autoepitopes, which have been reported in previous epitope mapping studies in patients.

Wet lab epitope mapping studies and our present *in silico* analysis have shown that the epitopes targeted by autoantibodies are localized within the NC1, NC2 and most probably the hinge region of the antigen [19-22]. Therefore, for measuring autoantibodies against collagen VII we have generated a chimeric protein containing all putative epitope-bearing regions of collagen VII, including its NC1 and NC2 domains and the hinge fragment. The chimeric protein, which was produced in a human cell line to ensure optimal posttranslational modifications, shows an increased yield and is more stable compared with the full-length collagen while containing all the epitopes/regions in one copy per molecule. Thus, its use as a substrate does not require preadsorption against bacterial proteins and ensures strict equimolar concentration of the NC1, NC2 and hinge regions.

Interestingly, our *in silico* analysis predicted the existence of both linear and conformational epitopes. Since immunoblotting is not quantitative and uses denatured antigen, to measure autoantibodies against both linear and conformational epitopes on collagen VII, we have used ELISA.

Possible isolated reactivity against hinge was reported so far only in 3 children with EBA [21] and was apparently present in our relatively large cohort of adult patients only in one patient. This resulted in a slightly lower specificity of the ELISA using the fused NC1 and NC2 domains when compared with the form containing NC1, hinge and NC2 in the present study. The percentage of EBA patients with autoantibodies targeting only epitopes outside its NC1 and NC2 domains is unknown. However, since EBA was generally previously diagnosed by reactivity with the NC1 domain of collagen VII, patients showing only reactivity against the hinge region may have been largely excluded from our study. Therefore, the immunossay described here allows characterizing the prevalence of autoantibodies against the hinge region of collagen VII and should increase the yield of EBA patients, which will test positive by ELISA. In addition, as suggested by previous studies [21,35], the reactivity against the hinge region of collagen VII may be associated with inflammatory rather than mechanobullous blistering disease. The new recombinant proteins generated in this study will help clarifying this aspect in larger cohorts of patients with disease associated with autoimmunity against collagen VII.

Low levels of collagen VII-specific autoantibodies were detected in a number of patients with other autoimmune

Table 2 Sensitivity and specificity of ELISA systems for the detection of autoantibodies against collagen VII

Study	Recombinant autoantigen	Commercially available	Sensitivity	Specificity	Reference
Chen *et al.* (n = 24)	NC1 domain	No	100%	-	[22]
Pendaries *et al.* (n = 41)	Collagen VII full length	No	68%	96%	[25]
Saleh *et al.* (n = 49)	NC1 + NC2	Yes	93.8%	98.1%	[26]
Komorowski *et al.*(n = 73)	NC1 domain	Yes	91.8%	99.8%	[27]
Licarete *et al.* (n = 50)	NC1-hinge-NC2	-	94%	98%	present study

blistering diseases and healthy subjects. In patients with autoimmune and inflammatory diseases the presence of autoantibodies against collagen VII may be the result of an epitope spreading process. Our findings are in line with the observation that healthy blood donors and patients without skin blisters show pemphigoid autoantibodies [36-38]. The pathogenic significance of collagen VII-specific autoantibodies in other autoimmune blistering diseases and in healthy subjects is still unclear. As shown in our present study, these autoantibodies do not show binding to the dermal-epidermal junction by indirect IF microscopy suggesting that they do not bind *in vivo*. In addition, our previous *in vivo* studies documented that the mere presence of tissue-bound autoantibodies in experimental EBA does not result in skin disease [11].

Autoantibodies with different molecular specificity are present in healthy individuals and in patients with various diseases (e.g., ANAs prevalence is about 3-15%). We have found collagen VII-specific autoantibodies in 4% and 18% of patients with CD and UC, respectively. Our present results in CD patients are in line with our previous study showing collagen VII-specific autoantibodies by immunoblotting in 5.8% of CD and 5.8% of UC patients, in contrast to over 60% of CD patients initially reported [23,24]. Interestingly, we measured collagen VII-specific autoantibodies in a higher percentage of UC patients compared with 5.8% and 12.9%, that were reported in the previous studies [39,40]. The reason for this discrepancy is not known and future studies in larger number of IBD patients should help defining the prevalence of collagen VII-specific autoantibodies in these patients. There is apparently no correlation of collagen VII-specific autoantibodies with inflammation markers in patients with CD and UC. While several hypotheses have been advanced, the induction of autoimmune response against collagen VII and the pathogenic significance of specific autoantibodies in inflammatory bowel disease is still elusive [2,41].

As with other autoantibody-induced diseases, ELISA levels of collagen VII-specific autoantibodies likely correlate with the disease severity [13,26]. It is therefore expected that measuring the autoantibody levels, will help predicting short-term clinical evolution in EBA patients and guide therapeutic decisions. A more general prognostic value of the levels of EBA autoantibodies for the long-term disease course, including the resistance to treatment, has not yet been addressed. Addressing this question, which requires a more wider approach in prospective clinical study, should be addressed in the future using the tools generated this study.

Our present results strongly suggest the existence of non-pathogenic autoimmunity against collagen VII in patients and healthy individuals. Why autoantibodies specific to collagen VII and XVII do not induce tissue damage in all individuals and conditions is not known. Inadvertent autoimmune responses may be uncoupled from disease by various mechanisms, including cryptic B cell autoepitopes, typically seen in Goodpasture syndrome [39,40], anatomic, cellular and molecular barriers that avert either tissue deposition of immune complexes [42,43] or the engagement of inflammatory effectors by tissue-bound antibodies [44]. In this context, the IgG subclass is a major determinant of autoantibody pathogenicity. Similar to a previously published report, our IgG subclass analysis revealed a dominant IgG4 response in patients with collagen VII-specific autoantibodies [45]. However, while the other subclasses of autoantibodies were less represented we could not document a strict restriction to IgG1 and IgG4 [45]. The mechanisms of tissue damage in EBA may be inflammatory, requiring the activation of complement and leukocytes by bound autoantibodies, or non-inflammatory just involving the binding of autoantibodies independent of their Fc portions [13]. Our own results and data from the literature show that in addition to IgG1, which shows good Fcγ-dependent complement- and leukocyte-activating capacity, non-complement-fixing IgG4 autoantibodies contribute to tissue damage by activating the leukocytes, albeit with a reduced efficiency compared with IgG1 [46]. In addition, IgG4 could induce tissue damage just by binding to collagen VII in an Fc-independent manner [13]. While experimental data to support this hypothesis are still scarce, our present findings suggest that IgG4 may unfold its pathogenic potential in this way.

Conclusions

In conclusion, we have developed an immunoassay using a chimeric recombinant collagen VII containing major *in silico* predicted and wetlab mapped autoepitopes for detecting autoantibodies against collagen VII. We show a low prevalence of collagen VII-specific autoantibodies in patients with unrelated inflammatory and autoimmune diseases. This immunoassay will be a useful tool for the sensitive and specific detection of collagen autoantibodies in epidermolysis bullosa acquisita and other diseases associated with autoimmunity against collagen VII.

Additional material

Additional file 1: Table S1 Predicted antigenic epitopes of human collagen VII.

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Authors' contributions
EL and CS designed and performed the ELISA, coordinated the data acquisition, analyzed and interpreted the data and drafted the manuscript. SG produced the recombinant protein, characterized its immunoreactivity and performed IgG subclass analysis by ELISA. MJR and CS performed the *in silico* analysis. GDZ, TH, MH, GZ, GH, JM, MFN and LBT provided serum samples used in the study and have participated in the experimental design and drafting of manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Definitions and outcome measures for bullous pemphigoid: Recommendations by an international panel of experts

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Our scientific knowledge of bullous pemphigoid (BP) has dramatically progressed in recent years. However, despite the availability of various therapeutic options for the treatment of inflammatory diseases, only a few multicenter controlled trials have helped to define effective therapies in BP. A major obstacle in sharing multicenter-based evidences for therapeutic efforts is the lack of generally accepted definitions for the clinical evaluation of patients with BP. Common terms and end points of BP are needed so that experts in the field can accurately measure and assess disease extent, activity, severity, and therapeutic response, and thus facilitate

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and advance clinical trials. These recommendations from the International Pemphigoid Committee represent 2 years of collaborative efforts to attain mutually acceptable common definitions for BP and proposes a disease extent score, the BP Disease Area Index. These items should assist in the development of consistent reporting of outcomes in future BP reports and studies. (J Am Acad Dermatol 2012;66:479-85.)

Key words: bullous pemphigoid; consensus; definitions; outcome measures; severity score.

CAPSULE SUMMARY

- It is impossible to compare the therapeutic outcomes from the majority of bullous pemphigoid studies using meta-analysis, as they have varying definitions and outcome measures.
- These recommendations, developed over the last 3 years by experts, provide appropriate definitions for the various stages of disease activity and therapeutic end points in bullous pemphigoid.
- These definitions can be used in case series and clinical trials to compare the efficacy of treatments for bullous pemphigoid.

Bullous pemphigoid (BP) is a common autoimmune bullous disease typically affecting the elderly. There have been only a handful of well-designed randomized controlled trials assessing the effectiveness of therapies for BP.[1] In relatively rare diseases where it is difficult to include enough patients to have sufficient power to compare different treatments, meta-analysis is a powerful tool that is used to pool data across trials. However, it is impossible to compare the therapeutic outcomes from the majority of these BP studies using meta-analysis, as they have varying definitions and outcome measures.

PURPOSE

The purpose of this statement is to provide appropriate definitions for the various stages of disease activity, define therapeutic end points in BP, and to propose an objective disease extent measure that can be used in clinical trials. The use of the same definitions and outcome measures makes the results of trials more comparable. Since definitions and outcome measures for pemphigus[2-4] have been published, most trials in pemphigus and reports have begun adopting these systems or referring to them when their existing trials using other measures were unable to show a difference.[5]

METHODS

An international BP definitions committee was organized in 2008, at the point when the international pemphigus definitions committee completed its similar work on pemphigus.[2] The committee was an expansion of the first committee and convened 7 times over 2 years to discuss the appropriate definitions. These meetings were held at the American Academy of Dermatology (AAD) annual meeting in San Antonio, TX, in 2009 (D. F. M. and V. P. W.); European Society for Dermatologic Research in Budapest, Hungary, in 2009 (D. F. M. and P. J.); the European Academy of Dermatovenereology in Berlin, Germany, in 2009 (D. F. M. and L. B.); the AAD in Miami, FL, in 2010 (D.F.M. and V. P. W.); the Pemphigus 2010 Meeting in Bern, Switzerland (V. P. W. and D. F. M.); and the International Pemphigus and Pemphigoid Meeting at the National Institutes of Health in November 2010 (V. P. W. and D. F. M.), in Bethesda, MD. The final meeting was held at the AAD in 2011 in New Orleans, LA (D. F. M. and V. P. W.). Meetings were supported in part by local dermatology societies. The draft definitions and end points were electronically mailed to the larger group, allowing for comments between meetings.

THE RECOMMENDATIONS

Observation points

The end points are illustrated and summarized (Fig 1 and Table I).

Early end points

"Baseline" is the point at which a physician starts treatment for BP.

"Control of disease activity" (disease control; beginning of consolidation phase) is defined as the point at which new lesions or pruritic symptoms cease to form and established lesions begin to heal. The time to disease control is the time between baseline and this control point.

"End of the consolidation phase" is defined as the time at which no new lesions or pruritic symptoms have developed for a minimum of 2 weeks and the majority (approximately 80%) of established lesions has healed. At this point tapering of corticosteroids often occurs. The length of the consolidation phase is the time between disease control and the end of consolidation phase.

Abbreviations used:

AAD:	American Academy of Dermatology
BP:	bullous pemphigoid
BPDAI:	Bullous Pemphigoid Disease Area Index
DAI:	Disease Area Index
PDAI:	Pemphigus Disease Area Index

"Transient lesions" are new lesions that heal within 1 week or pruritus lasting less than a week and clearing without treatment.

"Nontransient lesions" are new lesions that do not heal within 1 week or pruritus continuing more than a week with or without treatment.

Intermediate end points

During this period, the corticosteroids and other treatments are usually being tapered, but for some patients medication doses do not change because of flaring with attempts to taper treatment. "Complete remission during tapering" is the absence of nontransient lesions while the patient is receiving more than minimal therapy. There is no minimum time point here as the patient is under control but has not yet reached the desired outcome of disease remission on minimal or no therapy.

Late observation end points

Late observation end points of disease activity are identified as: (1) complete remission off therapy; and (2) complete remission on therapy, both of which only apply to patients who have had no new or established lesions for at least 2 months. "Complete remission off therapy" is defined as an absence of new or established lesions or pruritic symptoms while the patient is off all BP therapy for at least 2 months.

"Complete remission on therapy" is defined as the absence of new or established lesions or pruritus while the patient is receiving *minimal* therapy for at least 2 months. "Minimal therapy" is defined as less than or equal to 0.1 mg/kg/d of prednisone (or the equivalent) or 20 g/wk of clobetasol propionate and/or minimal adjuvant or maintenance therapy for at least 2 months, as shown in Fig 1 and discussed further below.

Minimal adjuvant therapy in BP corresponds to the following doses or less: methotrexate 5 mg/wk; azathioprine 0.7 mg/kg/d (with normal thiopurine s-methyltransferase level); mycophenolate mofetil 500 mg/d; mycophenolic acid 360 mg/d; or dapsone 50 mg/d. There has only been one small randomized controlled trial on tetracycline and niacinamide,[6] which was underpowered because of low numbers and was unable to demonstrate a difference. Nevertheless, the committee's expert opinion is that full therapeutic doses of the tetracyclines may work in localized forms of BP. As the tetracycline class of drugs is relatively nontoxic, the full therapeutic dose was listed among minimal therapies for BP.

"Partial remission off therapy" is defined as the presence of transient new lesions that heal within 1 week without treatment and while the patient is off all BP therapy for at least 2 months.

"Partial remission on minimal therapy" is defined as the presence of transient new lesions that heal within 1 week while the patient is receiving minimal therapy.

A newer term, "mild new activity," refers to fewer than 3 lesions a month (blisters, eczematous lesions, or urticarial plaques) that do not heal within 1 week, or the extension of established lesions or pruritus once per week but less than

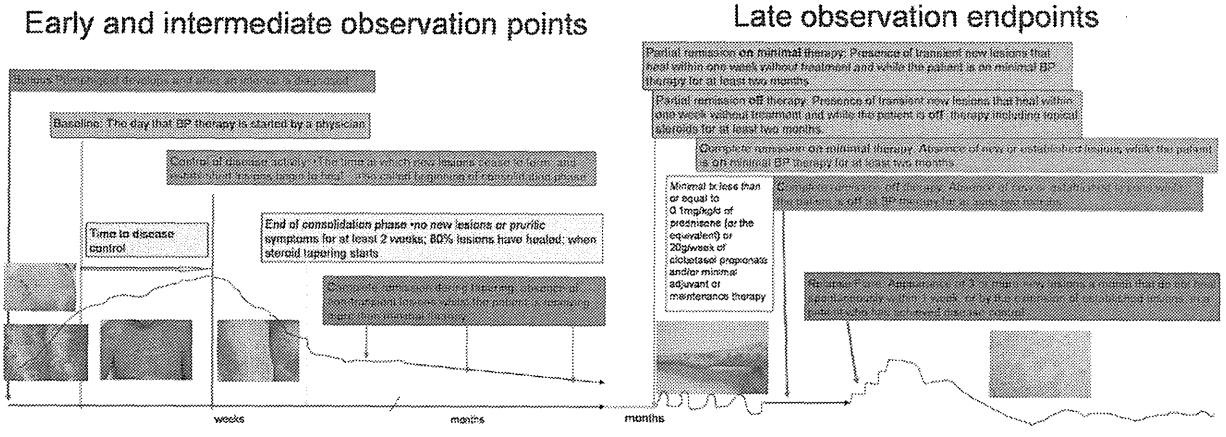

Fig 1. Pictorial depiction of end points in bullous pemphigoid.

Table I. Definitions for bullous pemphigoid

Early observation points	
Baseline	Day that BP therapy is started by physician
Control of disease activity	Time at which new lesions cease to form and established lesions begin to heal or pruritic symptoms start to abate
Time to control of disease activity (disease control; beginning of consolidation phase)	Time interval from baseline to control of disease activity
End of consolidation phase	Time at which no new lesions have developed for minimum of 2 wk and approximately 80% of lesions have healed and pruritic symptoms are minimal
Intermediate observation end points	
Transient lesions	New lesions that heal within 1 wk or pruritus lasting <1 wk and clearing without treatment
Nontransient lesions	New lesions that do not heal within 1 wk or pruritus continuing >1 wk with or without treatment
Complete remission during tapering	Absence of nontransient lesions while patient is receiving more than minimal therapy
Late observation end points	
Minimal therapy	≤ 0.1 mg/kg/d Of prednisone (or equivalent) or 20 g/wk of clobetasol propionate and/or minimal adjuvant or maintenance therapy
Minimal adjuvant therapy and/or maintenance therapy	Following doses or less: methotrexate 5 mg/wk; azathioprine 0.7 mg/kg/d (with normal thiopurine s-methyltransferase level); mycophenolate mofetil 500 mg/d; mycophenolic acid 360 mg/d; or dapsone 50 mg/d
Partial remission on minimal therapy	Presence of transient new lesions that heal within 1 wk while patient is receiving minimal therapy for at least 2 mo
Complete remission on minimal therapy	Absence of new or established lesions or pruritus while patient is receiving minimal therapy for at least 2 mo
Partial remission off therapy	Presence of transient new lesions that heal within 1 wk without treatment while patient is off all BP therapy for at least 2 mo
Complete remission off therapy	Absence of new or established lesions or pruritus while patient is off all BP therapy for at least 2 mo
Mild new activity	<3 Lesions/mo (blisters, eczematous lesions, or urticarial plaques) that do not heal within 1 wk, or extension of established lesions or pruritus once/wk but less than daily in patient who has achieved disease control; these lesions have to heal within 2 wk
Relapse/flare	Appearance of ≥ 3 new lesions/mo (blisters, eczematous lesions, or urticarial plaques) or at least one large (>10 cm diameter) eczematous lesion or urticarial plaques that do not heal within 1 wk, or extension of established lesions or daily pruritus in patient who has achieved disease control
Failure of therapy for initial control	Development of new nontransient lesions or continued extension of old lesions, or failure of established lesions to begin to heal or continued pruritus despite: Clobetasol propionate 40 g/d for 4 wk; or Prednisone 0.75 mg/kg/d equivalent for minimum of 3 wk with or without drugs used for maintenance therapy; or A tetracycline on full dosing for 4 wk; or Dapsone 1.5 mg/kg/d for 4 wk; or Methotrexate 15 mg/wk (if >60 kg and no major renal impairment) for 4 wk; or Azathioprine 2.5 mg/kg/d for 4 wk (if thiopurine s-methyltransferase level is normal); or Mycophenolate mofetil 40 mg/kg/d (if normal renal function, otherwise according to age/creatinine clearance) for 4 wk

BP, Bullous pemphigoid.

daily, in a patient who has achieved disease control. This term was not included in the pemphigus definitions but the committee thought that it might be important to capture this phase during studies to determine if some patients with BP and certain characteristics or treatments experienced new mild activity not significant enough to constitute a flare. In this way, it could be determined in the future if these patients with BP might benefit from a change of treatment plan or not.

Relapse/flare

The terms "relapse" and "flare" are used interchangeably and are defined as the appearance of 3 or more new lesions a month (blisters, eczematous lesions, or urticarial plaques) or at least one large (>10 cm diameter) eczematous lesion or urticarial plaque that does not heal within 1 week, or the extension of established lesions or daily pruritus in a patient who has achieved disease control.

Treatment failure

"Failure of therapy for initial control" is defined as the development of new nontransient lesions or continued extension of old lesions, or failure of established lesions to begin to heal or daily pruritus despite certain strengths of corticosteroids with or without higher doses of adjuvants. The dose of prednisone defined as treatment failure is 0.75 mg/kg/d equivalent for minimum of 3 weeks. This dose was selected because the Cochrane review of interventions for BP[1,7] determined that in acute BP there was no purpose in using prednisone at a higher dose than this. Topical clobetasol propionate at 40 g/d for 4 weeks was selected on the basis of the randomized controlled trials conducted by the French group.[8,9] Other therapies include tetracycline at full doses for 4 weeks; dapsone 1.5 mg/kg/d for 4 weeks; methotrexate 15 mg/wk (if >60 kg and no major renal impairment) for 4 weeks; azathioprine 2.5 mg/kg/d for 4 weeks (if thiopurine s-methyltransferase level is normal); or mycophenolate mofetil 40 mg/kg/d (if normal renal function, otherwise according to age/creatinine clearance) for 4 weeks. The definition does not imply these drugs and their respective doses are equivalent in therapeutic efficacy. Rather it provides a standardized agreement as to what can be defined as a failure of therapy.

BP disease activity index

Like the Pemphigus Disease Area Index (PDAI),[3] the BP Disease Area Index (BPDAI) measure has separate scores for skin and mucous membrane activity. Damage scores are separate as well and are included to remind physicians that not all visible lesions in BP represent active disease. Areas of the skin predominantly affected in BP[10] were taken into account when selecting the skin sites so that trials would better differentiate clinical response in BP. Hence, additional weighting was given to the arms and legs and less emphasis to the face and scalp, slightly different from the PDAI. The mucous membrane areas were retained from the PDAI even though it is relatively rare to see mucous membrane involvement in BP, so that the activity could be compared with extent of mucous membrane involvement in different autoimmune bullous diseases. There are separate columns for the extent of blistering and for the urticarial/eczematous lesions that may be more extensive in BP.

BPDAI PRURITUS COMPONENT - VAS

DATE: .....

☐ Baseline	☐ Begianing Consolidation
☐ Consolidation phase	☐ End of Consolidation
☐ Tapering phase	☐ Partial remission on minimal therapy
☐ Complete remission on minimal therapy	☐ Partial remission off therapy
☐ Complete remission off therapy	☐ Flare

A. How severe has your itching been over the last 24 hours?

0 1 2 3 4 5 6 7 8 9 10
None Severe

Score out of 10 =

B. How severe has your itching been the past week?

0 1 2 3 4 5 6 7 8 9 10
None Severe

Score out of 10 =

C. How severe has your itching been in the past month?

0 1 2 3 4 5 6 7 8 9 10
None Severe

Score out of 10 =

Average INTENSITY SCORE FOR PAST MONTH = (A+B+C) = /30

OR

For BP patients with impaired mental functioning:

☐	No evidence of itch (no excoriations)	0
☐	Mild itch (isolated excoriations up to two body sites)	10
☐	Moderate itch (excoriations on ≥ 3 body sites, impairment of daily activity	20
☐	Severe itch (generalized excoriation, sleep impairment)	30
	TOTAL SCORE	/30

Fig 2. Subjective Bullous Pemphigoid (*BP*) Disease Area Index (*BPDAI*) pruritus score. *VAS*, Visual analog scale.

As a major symptom that may herald the onset and recurrence of BP is pruritus, a separate subjective component of the BPDAI is proposed to measure the severity of this (Fig 2). Naturally, other causes of pruritus in the elderly must be excluded, such as xerosis, dermatitis, renal impairment, liver impairment, and scabies. Providing that only pruritus related to BP is considered in the definitions and scored, this system can be used to subjectively grade the intensity of pruritus using a visual analog scale to answer the question, "How severe is your itching today?" and the patient marks an "x" on the 0- to 10-cm line where 0 is no itch and 10 is maximal itching. The degree of itching is measured as the distance in centimeters from 0, out of 10. This is repeated for the severity overall of itching in the past week and month. A total score is calculated from this out of 30. If the patient with BP is incapable of completing a reliable visual analog scale rating, for example, as a result of dementia, then the degree of pruritus is inferred, based on the extent of excoriations alone, also scored

BPDAI					
SKIN	ACTIVITY		ACTIVITY		DAMAGE
Anatomical location	Erosions/Blisters	Number of Lesions if <3	Urticaria/ Erythema / Other	Number of Lesions if <3	Pigmentation / Other
	0 absent		0 absent		Absent 0, present 1
	1 1-3 lesions, none > 1 cm diameter		1 1-3 lesions, none >6 cm diameter		
	2 1-3 lesions, at least one > 1 cm diameter		2 1-3 lesions, at least one lesion > 6 cm diameter		
	3 >3 lesions, none > 2 cm diameter		3 >3 lesions, or at least one lesion > 10 cm		
	5 >3 lesions, and at least one >2 cm		5 >3 lesions and at least one lesion > 25 cm		
	10 >3 lesions, and at least one lesion >5 cm diameter or entire area		10 >3 lesions and at least one lesion > 50 cm diameter or entire area		
Head					
Neck					
Chest					
Left arm					
Right arm					
Hands					
Abdomen					
Genitals					
Back/Buttocks					
Left leg					
Right leg					
Feet					
Total skin	/120		/120		
MUCOSA	Erosions/Blisters				
	1 1 lesion				
	2 2-3 lesions				
	5 >3 lesions, or 2 lesions >2cm				
	10 entire area				
Eyes					
Nose					
Buccal mucosa					
Hard palate					
Soft palate					
Upper gingiva					
Lower gingiva					
Tongue					
Floor of Mouth					
Labial Mucosa					
Posterior Pharynx					
Anogenital					
Total Mucosa	/120				

Fig 3. Objective bullous pemphigoid disease area index

out of 30 (Fig 2). This subjective itch score will not be combined with the objective part of the BPDAI (Fig 3). Eventually, a quality-of-life tool for BP will be necessary as well. The BPDAI will be undergoing validation studies, similar to the partial validation done thus far with the PDAI.[3]

DISCUSSION AND CONCLUSION

Despite many trials evaluating therapeutic options for BP, it has been difficult to compare the results from these trials because of the large number of end points and definitions of disease. The formation of an international committee of bullous

disease experts able to meet face to face on a regular basis has provided a mechanism for developing agreement on these issues for BP. This statement with agreed-upon common definitions, and the ongoing discussion and refinement of proposed common measurements for patients with BP, are the initial and necessary steps toward progress in the clinical evaluation and therapy of BP. Further progress and advancement will require a continued unified effort.

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Research Article

Distinct Characteristics in Japanese Dermatitis Herpetiformis: A Review of All 91 Japanese Patients over the Last 35 Years

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We reviewed all 91 Japanese dermatitis herpetiformis (DH) patients reported over the last 35 years. The male-to-female ratio was 2 : 1. The mean age at onset was 43.8, and 13 years earlier for female patients. More than half of these Japanese DH patients showed granular IgA deposition in the papillary dermis, and another one-third showed fibrillar IgA deposition. The male patients with granular IgA deposition were 10 years older than those with fibrillar deposition. Whereas patients with granular IgA deposition showed typical distribution of the skin lesions, the predilection sites of DH tended to be spared in patients with fibrillar IgA deposition. Only 3 patients had definite gluten-sensitive enteropathy. There was a statistical difference in the frequency of human leukocyte antigen (HLA)-DR9 between the granular group and controls among Japanese. No patients had HLA-DQ2 or -DQ8, which is frequently found in Caucasian DH patients. The absence of HLA-DQ2/DQ8, the inability to identify celiac disease in most cases, the predominance of fibrillar IgA, and the unusual distribution of clinical lesions in Japanese patients suggest that Japanese DH may be a subset of DH patients and have a pathogenesis which is different from that currently proposed in Caucasian DH patients.

1. Introduction

Dermatitis herpetiformis (DH) is a rare, intensely pruritic, chronic and recurrent papulovesicular disease, in which the lesions usually develop symmetrically on the extensor surfaces. This disease can be clearly distinguished from other subepidermal blistering diseases by histopathological and immunological criteria. Biopsy of an early lesion shows collections of neutrophils at the papillary tips, and direct immunofluorescence (DIF) reveals nonlinear (mostly granular, or fibrillar) IgA deposition in the papillary dermis.

DH is most prevalent among the Caucasian population, and several population-based studies have been conducted, which disclosed a close association with gluten-sensitive enteropathy (GSE) and the human leukocyte antigen (HLA)-DQ2 or HLA-DQ8 [1–5]. In contrast, only case reports and one review article have been published in Japan, reflecting rare occurrence of DH in Japan [6–85]. The previous review of Japanese DH cases revealed differences from Caucasian DH, such as a high frequency of fibrillar IgA deposition in the papillary dermis, a rarity of GSE, and the absence of HLA-B8/DR3/DQ2 haplotype [59].

The fibrillar immunofluorescence pattern of IgA deposition in DH was hypothesized to be related to longitudinal sectioning of affected dermal microfibril bundles, while the granular pattern represents transverse sectioning. However, confocal laser-scanning microscopy revealed numerous fibrils stained with anti-IgA antiserum, extending from the dermoepidermal junction to 50 to 110 μm deep in the dermis. They crossed each other at various angles to form a three-dimensional network. Moreover, immune electron microscopy demonstrated the diffuse dispersion of immune deposits on the surface of microfibrils of dermal microfibril bundles [86]. These findings signify that fibrillar IgA deposition is a distinct pattern. Although fibrillar IgA deposition

in DH is ignored in some review articles of DH [87–89], it cannot be dismissed if DH is to be understood sufficiently.

To disclose the unique features of Japanese DH, we have reviewed all reports of Japanese DH patients from 1976 to 2011, most of which were written in Japanese [6–85]. We also compare the characteristics of patients with granular IgA deposition to those with fibrillar IgA deposition.

2. Materials and Methods

First we selected Japanese DH cases by searching Ichushi Web (ver. 5), a Japanese medical literature database provided by NPO Japan Medical Abstracts Society, using the term, "dermatitis herpetiformis Duhring" in Japanese, and PubMed using the term, "dermatitis herpetiformis AND Japanese." Then, we also collected all articles for Japanese DH cited by these articles. Eventually, more than 200 articles were collected. Since earlier Japanese reports of DH included linear IgA bullous dermatosis cases, we omitted these cases. Thus, we selected only cases, which showed subepidermal blisters, neutrophilic microabscesses, and nonlinear IgA deposition in the papillary dermis. Finally, 91 Japanese DH cases reported from 1976 to 2011 were accumulated [6–85].

Because one of the characteristics of Japanese DH is a high frequency of fibrillar IgA deposition, we compared the cases with granular IgA deposition (granular group) and those with fibrillar IgA deposition (fibrillar group). We performed Student's t-test for comparison of age distribution, and the χ^2 test for the HLA study using the SPSS software (ver. 19). A P value of less than 0.05 was considered to indicate statistical significance. P values for the HLA study were corrected by multiplying the P value by the number of antigens tested (HLA-DR = 10).

3. Results

3.1. Overview of Japanese DH (Table 1). Ninety-one Japanese DH patients consisted of 61 males aged between 1 and 87 years (mean 51.5 years, SD 20.5) and 30 females aged between 18 and 72 years (mean 36.8 years, SD 14.1). The data on the age at onset of DH were available for 48 males (1–87 years, mean 48.5 years, SD 19.6) and 27 females (14–72 years, mean 35.3 years, SD 13.0). The female patients started suffering from DH 13 years earlier than the male patients. No patients had any family history of DH or celiac disease (CD).

Clinical manifestation was polymorphic, consisting of erythemas, urticarial plaques, papules, and herpetiform vesicles and blisters. Superficial erosions and excoriation due to scratching were also frequently noted. Most patients presented intense pruritus, being mild in other patients. More than half Japanese DH patients had lesions on the predilection sites as in Caucasian DH, that is, the elbow, buttock, knee, face, ear, neck, scalp, and groin. In particular, 44% of Japanese DH patients had lesions on the elbow, buttock, and/or knee. The face, ear, neck, scalp, and groin were affected in only a few patients. Interestingly, 41 and 55 Japanese DH patients presented skin lesions on nonpredilection sites such as the extremities and trunk, respectively, with or without concurrent lesions on predilection sites. Six patients had lesions on the whole body. No mucosal involvement was reported.

Most biopsy specimens showed subepidermal blisters and an accumulation of neutrophils with or without a few eosinophils at the papillary tips. In DIF, 50 (54.9%) cases showed granular IgA deposition (referred as granular group), and 33 (36.3%) cases showed fibrillar IgA deposition in the papillary dermis (referred as fibrillar group). Seven cases showed both granular and fibrillar IgA depositions, and only one case showed cluster IgA deposition [80]. Twenty (22.0%) cases showed C3 deposition, and 9 (9.9%) cases showed IgG deposition in the papillary dermis. No circulating antibodies to the basement membrane zone were shown in the cases for whom indirect immunofluorescence (IIF) results were available.

Gluten-sensitive enteropathy (GSE) was associated with only 3 cases, who responded to gluten-free diet (GFD) with dapsone [24, 47, 59]. However, GFD for one case was not strict, and no information about long-term strict GFD was obtained for another 2 cases. While jejunum biopsy revealed villous atrophy in 3 patients including 1 patient with GSE [14, 39, 59], other 3 patients with no clinical symptoms of gluten sensitivity did not show any change [24, 33, 52].

Eight cases had diabetes mellitus (DM). One of those had noninsulin-dependent type DM, and four also seemed to have noninsulin-dependent type DM according to their therapy. The type of DM of three cases was unknown. Three cases had lymphoma. One case each had mycosis fungoides and anaplastic large cell lymphoma although the type of lymphoma was unknown in one case [47, 52]. Thyroid disease and Sjögren syndrome were also found in one each case of Japanese DH [9, 59].

The most common HLA antigen found in Japanese DH was Cw3, followed by A2, DR9, A24, and DR4 in the descending order. Compared with the controls Japanese population [90], there was no increase in the frequencies of HLA class I antigens (A, B, and C antigens), whereas there was a slightly increased frequency of HLA-DR9 in all the DH patients examined for HLA. No patient had either HLA DQ2 or DQ8.

Antireticulin, antigliadin and antiendomysial antibodies were investigated in small number of Japanese DH cases, and none had these antibodies. IgA antitransglutaminase antibodies have been reported in only 2 Japanese DH patients. In both cases, antiepidermal transglutaminase (eTG) antibodies were detected, while antitissue transglutaminase (tTG) antibodies were not [84].

Dapsone was effective for most patients. Although most patients treated with dapsone required reduced dosage of dapsone for maintenance therapy, the lesions of 5 patients were completely cleared, and no therapy was required after several-month administration of dapsone without any other treatment [41, 44, 46, 64, 78]. The efficacy of GFD was difficult to evaluate, particularly, in patients without clinical symptoms of GSE because they had dapsone administration concurrently, and GFD was not strict at all [13, 19, 20]. In contrast, GFD seemed to relieve abdominal symptoms in the patients with GSE although GFD for one patient was not strict [24, 47, 59]. Topical steroid was sufficient to cure the lesions completely in some patients. In one case

Table 1: Clinical characteristics of 91 patients.

	N or age/N of data available	
Gender		
Male	61/91	
Female	30/91	
Age at the initial visit, mean ± SD (range), years	46.6 ± 19.9/91	(1–87)
Male	51.5 ± 20.5/61	(1–87)
Female	36.8 ± 14.1/30	(18–72)
Age at onset, mean ± SD (range), years	43.8 ± 18.6/75	(1–87)
Male	48.5 ± 19.6/48	(1–87)
Female	35.3 ± 13.0/27	(14–72)
Site of lesion		
Elbow	33/84	(39.3%)
Knee	29/84	(34.5%)
Buttock	30/84	(35.7%)
Elbow and/or knee and/or buttock	37/84	(44.0%)
Face	11/84	(13.1%)
Ear	9/84	(10.7%)
Neck	8/84	(9.5%)
Scalp	6/84	(7.1%)
Groin	4/84	(4.8%)
At least one predilection site	49/84	(58.3%)
Extremities*	41/84	(48.8%)
Trunk**	55/84	(65.5%)
Whole body***	6/84	(7.1%)
IgA deposition in the papillary dermis		
Granular	50/91	(54.9%)
Fibrillar	33/91	(36.3%)
Granular and fibrillar	7/91	(7.7%)
Cluster	1/91	(1.1%)
Other deposition in the papillary dermis		
C3	20/91	(22.0%)
IgG	9/91	(9.9%)
IgM	4/91	(4.4%)
Fibrinogen	2/91	(2.2%)
GSE	3/91	(3.3%)
Jejunum mucosa biopsy		
Villous atrophy****	3/6	(50.0%)
No change*****	3/6	(50.0%)
Associated diseases		
Diabetes mellitus	8/91	(8.8%)
Lymphoma	3/91	(3.3%)
Thyroid disease	1/91	(1.1%)
Sjögren syndrome	1/91	(1.1%)
HLA antigen§		
DR4	13/31	(41.9%)#
DR9	15/31	(48.4%)##

Table 1: Continued.

	N or age/N of data available	
Other antibodies		
Antireticulin antibodies	0/9	(0.0%)
Antigliadin antibodies	0/3	(0.0%)
Antiendomysial antibodies	0/3	(0.0%)
Anti-tTG IgA antibodies	0/2	(0.0%)
Anti-eTG IgA antibodies	2/2	(100.0%)
Therapy		
Dapsone	62/82	(75.6%)
Dapsone + gluten-free diet	7/82	(8.5%)
Gluten-free diet + topical steroid	1/82	(1.2%)
Topical steroid	9/82	(11.0%)
Others[†]	3/82	(3.7%)

*Not including cases limited only to elbow or knee; **not including cases limited only to buttock, neck, or groin; ***not including cases limited to combination of predilection sites; ****including 1 patient with GSE; *****no patients had GSE; GSE: gluten-sensitive enteropathy; tTG: tissue transglutaminase, eTG: epidermal transglutaminase; [†]including minocycline and topical steroid, salazosulfapyridine, and zinc oxide ointment; [§]frequency in HLA antigens of control was depicted from [90]. [#]$P = 0.9$, [##]$P = 0.007$, corrected $P = 0.07$.

the lesions disappeared 4 months after tonsillectomy [51]. Except for the 3 patients with GSE, no patients developed clinical symptoms of gluten sensitivity throughout the course although they were taking a normal diet.

3.2. Comparison of Granular and Fibrillar Groups (Table 2). The number of cases in the granular group was approximately 1.5 times higher than that in the fibrillar group. In both groups, the male patients were twice the number of the female patients. The mean age at onset of male patients in the granular group was almost 10 years older than that in the fibrillar group although no statistical significance was obtained. The mean ages at onset of female patients in both groups were relatively close. The mean ages at onset of the male and female patients were relatively close in the fibrillar group, while the mean age at onset of the male patients was 15 years older than that of the female patients in the granular group.

Patients in the granular group had lesions on the elbow, knee, buttock, face, ear, neck, scalp, and/or groin, which were common sites in the Caucasian DH patients, more frequently than those in the fibrillar group. Particularly, patients in the granular group had lesions on the elbow, knee, and/or buttock almost three times as frequently as those in the fibrillar group. C3 deposition was more frequently seen in the granular group than the fibrillar group. Comparison for small bowel disease and associated diseases was difficult because of a small number of cases involving these diseases in both groups. Although there were no statistical differences in the frequency of the HLA type between the granular and the fibrillar groups, a statistical difference in the frequency of HLA-DR9 between the granular group and the controls was found (corrected $P = 0.02$).

4. Discussion

The mean age of Japanese DH patients at the initial visit was 46.6, with a male predominance of 2 : 1. The age range and the male-to-female ratio in Japanese DH study are very similar to those in the Caucasian study [5, 91, 92]. Caucasian female patients with DH also tend to develop skin lesions at a younger age than male patients [5, 92]. Although a high incidence of familial DH has been reported in Caucasian patients [93, 94], no family history was found in Japanese patients. The clinical manifestation and distribution, as well as histopathological features, were also similar to those found in Caucasians [95, 96].

The distinct results of DIF in Japanese DH were noteworthy. More than one-third (36.3%) of Japanese DH patients showed fibrillar IgA deposition in the papillary dermis. In contrast to the result in a previous review of Japanese DH, which pointed out a higher frequency of fibrillar IgA deposition than that of granular IgA deposition [59], our study revealed that most common IgA deposition in Japanese DH was the granular pattern. However, the frequency of fibrillar IgA deposition is still high, when compared to that in Caucasian DH [97].

When compared between the granular and fibrillar groups, male patients in the granular group were older than those in fibrillar group although apparent statistical significance was not obtained. The lesions in the fibrillar group seemed to spare the predilection sites of DH, such as elbow, knee, and buttock, while the lesions in the granular group frequently involve these sites.

Recently cases with combined granular and fibrillar IgA deposition were reported although it is not clear whether this combined deposition is just extraordinary or incidental findings or not [17, 19, 41, 49, 71, 79, 81]. Only one case showed cluster IgA deposition although detailed data were not available [80]. The results of IIF studies showed no specific IgA antibodies. This was different from Caucasian results, which showed 63.5% positivity against the endomysium of smooth muscle [92].

In Caucasian patients, DH has a clear relationship to CD and is considered to be the cutaneous expression of