References

1 Demitsu T, Kakurai M, Yoneda K *et al.* Localized pemphigoid (Brunsting-Perry type) with IgG antibody to BP180 NC16a domain resembling lupus erythematosus successfully treated with topical tacrolimus therapy. *J Eur Acad Dermatol Venereol* 2009; **23**: 79-80.

2 Takeichi S, Kubo Y, Arase S *et al.* Brunsting-Perry type localized bullous pemphigoid, possibly induced by furosemide administration and sun exposure. *Eur J Dermatol* 2009; **19**: 500-3.

3 Tanaka N, Dainichi T, Ohyama B *et al.* A case of epidermolysis bullosa acquisita with clinical features of Brunsting-Perry pemphigoid showing an excellent response to colchicine. *J Am Acad Dermatol* 2009; **61**: 715-9.

4 Joly P, Ruto F, Thomine E *et al.* Brunsting-Perry cicatricial bullous pemphigoid: a clinical variant of localized acquired epidermolysis bullosa? *J Am Acad Dermatol* 1993; **28**: 89-92.

5 Murata Y, Tani M, Kumano K. Localized chronic pemphigoid of Brunsting-Perry. Ultrastructural localization of IgG and complement components. *Arch Dermatol* 1983; **119**: 921-4.

6 Honoki K, Muramatsu T, Nakatani C *et al.* A case of nonscarring inflammatory epidermolysis bullosa acquisita: characterization of IgG autoantibodies by immunofluorescence, immunoblotting and immunogold electron microscopy. *J Dermatol* 1998; **25**: 666-72.

7 Jones JC, Hopkinson SB, Goldfinger LE. Structure and assembly of hemidesmosomes. *Bioessays* 1998; **20**: 488-94.

8 Hashimoto T, Dainichi T, Ohyama B *et al.* A case of antilaminin 332 mucous membrane pemphigoid showing a blister on the bulbar conjunctiva and a unique epitope on the alpha3 subunit. *Br J Dermatol* 2010; **162**: 898-9.

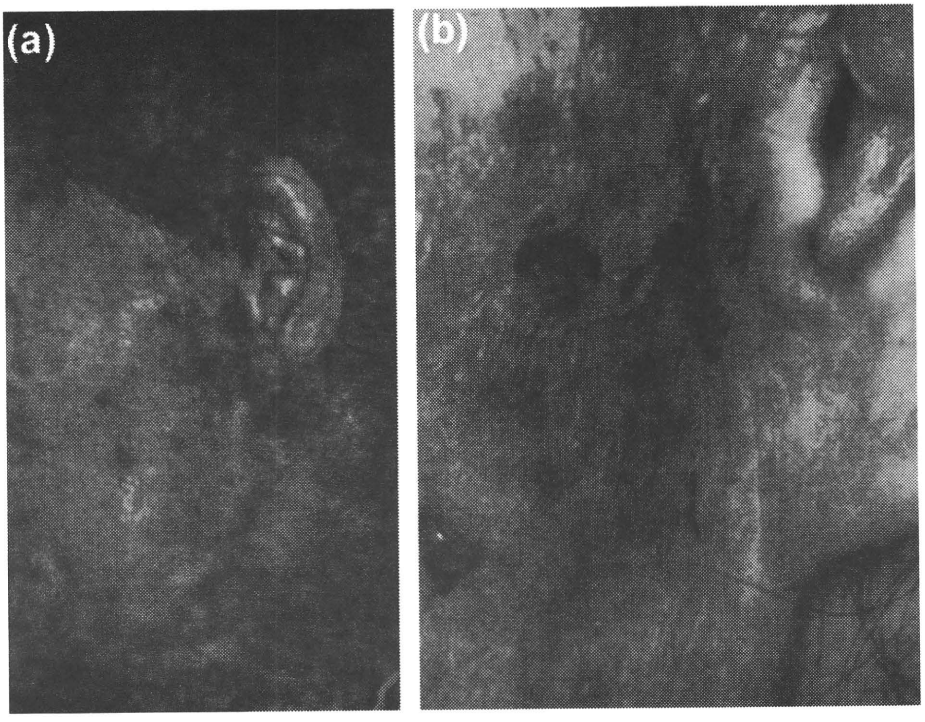
9 McMillan JR, Shimizu H. Desmosomes: structure and function in normal and diseased epidermis. *J Dermatol* 2001; **28**: 291-8.

10 Hashimoto T, Hamada T, Dainichi T *et al.* How does intramolecular epitope spreading occur in BPAG2 (BP180)? *J Invest Dermatol* 2010; **130**: 924-6.

Figure legends

Fig 1. (a) Erythemas, erosions and small vesicles limited to the left preauricular region. (b) Scar formation was also observed on the epithelialized old skin lesions.

Fig 2. (a) Haematoxylin and eosin-stained section revealing subepidermal bulla. (b),(c) Direct immunofluorescence showing IgG (b) and C3 (c) deposition to BMZ (arrows). (d) Indirect immunofluorescence revealing IgG autoantibodies reactive with both keratinocyte cell surface and BMZ (original magnifications: ×200 (a), ×200 (b), ×400 (c) and ×400 (d). (e) Immunoblotting, using human epidermal extract showing reactivity with the 210 kDa and 250 kDa desmoplakins I and II, as well as the 230 kDa BP230. Immunoblotting using BP180 NC16a recombinant protein (RP) showing no reactivity. Immunoblotting using purified laminin-332 showing reactivity with the 140 kDa β3 and the 105 kDa γ2 subunits of laminin-332. PV: pemphigus vulgaris. PNP: paraneoplastic pemphigus. BP: bullous pamphigoid. DPL: desmoplakin. MMP: mucous membrane pemphigoid.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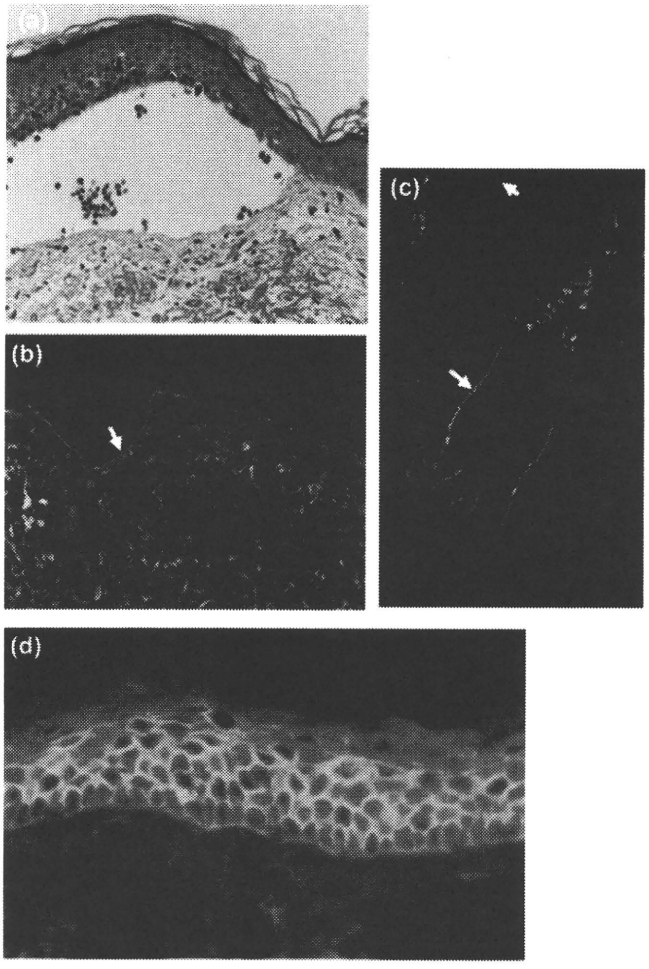
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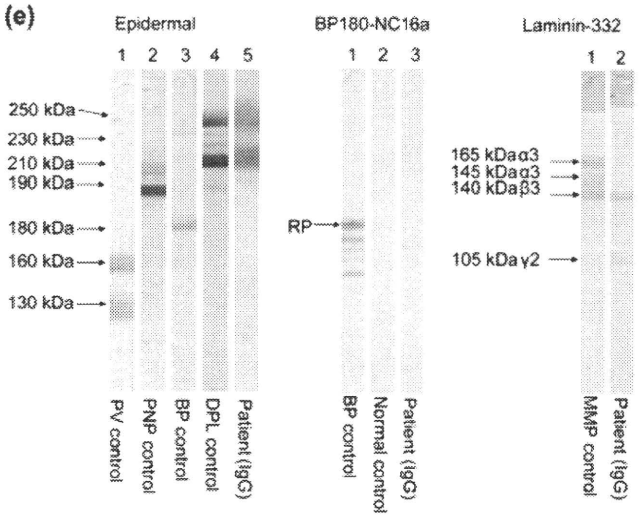
(e)
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230 kDa
210 kDa
190 kDa
180 kDa
160 kDa
130 kDa
PV control
PNP control
BP control
DPL control
Patient (IgG)
BP180-NC16a
1 2 3
RP
BP control
Normal control
Patient (IgG)
Laminin-332
1 2
165 kDaα3
145 kDaα3
140 kDaβ3
105 kDaγ2
MMP control
Patient (IgG)

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Development of NC1 and NC2 domains of Type VII collagen ELISA for the diagnosis and analysis of the time course of epidermolysis bullosa acquisita patients

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ABSTRACT

Background: Epidermolysis bullosa acquisita (EBA) is an acquired autoimmune mechanobullous disease. EBA patients possess autoantibodies against type VII collagen which is composed of a collagenous domain flanked by non-collagenous NC1 and NC2 domains. It was reported that major epitopes reside within the NC1 domain and minor epitopes reside within NC2 domain.
Objective: The aim of this study is to develop a sensitive and specific ELISA to facilitate the diagnosis of EBA.
Methods: We developed ELISAs using recombinant NC1 domain produced by mammalian expression system and recombinant NC2 domain produced by mammalian or bacterial expression system to characterize autoantibodies in EBA. Next, we developed an ELISA using a combination of the NC1 (mammalian expression) and NC2 (bacterial expression). We tested the ELISAs with 49 EBA sera, 55 normal control sera, 20 pemphigus vulgaris and 20 bullous pemphigoid sera.
Results: When we evaluated the 49 EBA sera using the NC1 and NC2 ELISAs, 38 (77.5%) reacted with NC1 domain only, 7 sera (14.2%) reacted with both NC1 and NC2 domains, and one serum (2%) reacted with NC2 domain only. Therefore, to increase the sensitivity of the assay, we developed an ELISA coated with a mixture of recombinant NC1 and NC2 domains, resulting in 93.8% sensitivity and 98.1% specificity. By analyzing the time course of two EBA patients, ELISA scores fluctuated in parallel with their disease activity.
Conclusion: We conclude that the NC1 + NC2 ELISA can be a practical assay for the diagnosis and follow up of the antibody titers of EBA patients.
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1. Introduction

Epidermolysis bullosa acquisita (EBA) is an acquired mechanobullous disease characterized by the presence of autoantibodies against type VII collagen. The etiology of EBA is unknown, however an autoimmune pathogenesis is suggested by the demonstration of IgG deposits at the dermo-epidermal junction of EBA patients by immunofluorescence [1–4].

The diagnostic clinical criteria for EBA are trauma-induced blisters that heal with milia and scarring in the absence of family history of epidermolysis bullosa. The immunopathological criteria are subepidermal blisters and linear deposits of IgG in the basement membrane zone (BMZ). The demonstration of IgG autoantibodies labeling the dermal side of salt-split skin is the most specific diagnostic assay [5].

Type VII collagen is a structural component of anchoring fibrils which stabilize the dermo-epidermal adherence. Type VII collagen is composed of central collagenous three identical alpha helical chains. Each alpha chain is flanked on one side by a 145 kDa amino-

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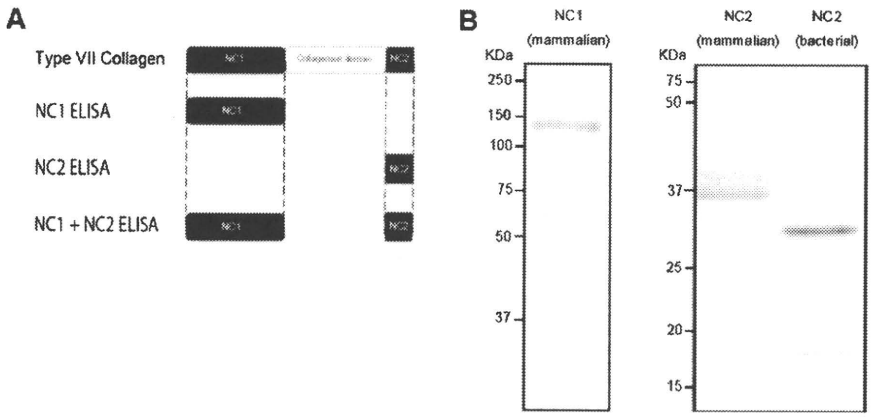

Fig. 1. Recombinant proteins used for the ELISAs. (A) Schematic diagram of the recombinant proteins used for the ELISAs. Type VII collagen, which consists of a 145 kDa amino-terminal non collagenous domain (NC1), 145 kDa central collagenous triple-helical domain and a 34 kDa carboxyl-terminal noncollagenous domain (NC2). NC1 ELISA used recombinant NC1 domain produced by mammalian expression system. NC2 ELISA used recombinant NC2 domain produced by mammalian expression system or bacterial expression system. NC1 + NC2 ELISA used a 2:1 weight mixture of recombinant NC1 produced by mammalian expression and recombinant NC2 produced by bacterial expression systems. (B) Purified recombinant NC1 and NC2 domains analyzed by Coomassie blue staining. Purified recombinant NC1 domain produced by mammalian expression system was run on 7.5% SDS–PAGE. Purified recombinant NC2 domain produced by mammalian expression system and purified recombinant NC2 domain produced by bacterial expression system were run on 15% SDS–PAGE. The molecular weight of NC1, NC2 (mammalian) and NC2 (bacterial) were 145 kDa, 34 kDa and 27 kDa, respectively.

terminal non-collagenous domain (NC1) and on the other side by a 34 kDa carboxyl-terminal noncollagenous domain (NC2) (Fig. 1A). Within the extracellular space type VII collagen forms an antiparallel tail-to-tail dimers stabilized by disulphide bonding at NC2 region. The antiparallel dimers aggregate laterally to form globular NC1 domains at both ends of the structure [6–8].

The major antigenic epitopes of type VII collagen are located within the NC1 domain of type VII collagen [9,10]. NC2 domain of type VII collagen contains minor antigenic epitopes [11,12]. Moreover, some EBA cases bind to the central collagenous domain of type VII collagen [13–15].

Conventional assays used for the diagnosis of EBA are direct immunofluorescence (IF) using the patient's own biopsy, indirect IF using the patient's own serum, salt-split skin IF and immunoblot analysis using dermal extract. Direct and indirect IF cannot differentiate EBA from bullous pemphigoid (BP) because IF shows linear deposits of IgG in the BMZ in both diseases. Salt-split skin IF is highly specific for the diagnosis of EBA [5,16], however it is not routinely performed in many centers. Immunoblot cannot detect autoantibodies against conformation-dependent epitopes because it uses denatured and reduced antigens as substrates.

In this study, we sought to develop a practical ELISA to detect autoantibodies against type VII collagen for the diagnosis of EBA. Several different ELISAs have been reported previously for characterizing autoantibodies in EBA [9,10,12,17]. However the previous ELISAs utilized only the NC1 domain or the subdomain of the NC1 of type VII collagen. Here we evaluated autoantibodies in EBA using NC1 domain ELISA and NC2 domain ELISA and found that small percentage of EBA sera bound exclusively to the NC2 domain. Therefore, we developed an ELISA using a combination of the NC1 and NC2 domains of type VII collagen and evaluated the usefulness of the ELISA for the diagnosis and monitoring of the disease activity in EBA.

2. Materials and methods

2.1. Patients and sera

Serum samples were obtained from 49 EBA patients. All patients examined in this study had typical clinical and histological features of EBA. The diagnosis of all patients was confirmed by detection of IgG deposits at the dermal side of dermo-epidermal junction by indirect IF of salt-split skin. Healthy control sera were obtained from 55 normal subjects. Disease control sera were obtained from 20 patients with BP and 20 patients with pemphigus vulgaris. The diagnosis was confirmed by the clinical criteria, routine histology and IF. All sera were stored at −30 °C until assayed.

2.2. Production and purification of recombinant NC1 and NC2

2.2.1. Preparation of recombinant NC1 domain produced by mammalian expression system

The coding sequence of NC1 domain of type VII collagen was generated by RT-PCR amplification using cDNA from human amniotic epithelial cells (WISH cells) as template, 5′-ATGACGCTGCGGCTTCTGGT-3′ as the forward primer and 5′-CTTTGGACAATACACTGGGC-3′ as the reverse primer. The RT-PCR product was inserted in pCR2.1 vector of TA-cloning kit (Invitrogen, Carlsbad, CA) and the DNA fragment was sequenced. The sequence coincided with the NC1 domain gene of human Type VII collagen (GenBank accession no. L02870). The NC1 DNA fragment was subcloned into the *Hind*III–*Eco*RI site of the mammalian expression pSecTag2 version E vector (Invitrogen, Carlsbad, CA). This subcloned vector was used to express the recombinant NC1 domain with myc-tag and 6×His-Tag at the C-terminus in Chinese hamster ovary cells (CHO cells).

CHO cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The NC1 expression vector was transfected into CHO cells using TransIT-CHO Transfection kit (Mirus, Madison, WI). The transfected CHO cells were single-cloned by the limiting dilution method under selection with culturing in 0.5 mg/ml Zeocin-containing DMEM medium supplemented with 10% FBS. The selected clonal cell line was cultured in 0.2 mg/ml Zeocin-containing CHO-S-SFMII (Invitrogen, Carlsbad, CA) of serum free medium for one week. The supernatant of culture media containing the NC1 recombinant protein were concentrated 10-fold by Amicon Stirred Ultrafiltration cell with 10,000 Ultrafiltration membranes (Millipore, Bedford, MA) and applied on a Talon cobalt-chelate column (Clontech, Palo Alto, CA). The yield of the purified NC1 recombinant protein was about 5.3 mg against 1 l of serum free medium. The purified NC1 recombinant protein was subjected to 7.5% SDS–PAGE analysis. The analysis confirmed that the purified NC1 protein was 145 kDa.

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2.2.2. Preparation of recombinant NC2 domain produced by mammalian expression system

The coding sequence of the NC2 domain of type VII collagen was generated by the RT-PCR amplification using cDNA from WISH cells as template, 5′-GCACTGACGGAGGATGACATC-3′ as the forward primer and 5′-GTCCTGGGCAGTACCTGTCCC-3′ as the reverse primer. The RT-PCR product was inserted in pCR2.1 vector of TA-cloning kit (Invitrogen, Carlsbad, CA) and the inserted DNA fragment was sequenced. The sequence coincided with the NC2 domain gene of human Type VII collagen (GenBank accession no. L02870). The NC2 DNA fragment was subcloned into *Hind*III–*Eco*RI site of the mammalian expression pSecTag2 version E vector (Invitrogen, Carlsbad, CA). This subcloned vector was used to express the recombinant NC2 domain with myc-tag and 6 × His-Tag at the C-terminus in Chinese hamster ovary cells (CHO cells).

CHO cells stably expressing recombinant NC2 domain were obtained as mentioned above. First we tried to produce recombinant NC2 domain cultured in serum-free medium as we did for recombinant NC1 domain but we failed to produce it for unknown reason. Therefore we cultured the CHO cells in the 10% FBS supplemented DMEM containing 0.1 mg/ml Zeocin. The NC2 recombinant protein was purified in two steps. First, it was purified using His tagged Protein purification Kit (MBL, Nagoya, Japan) from the filtrated media, and then was passed through Protein A Sepharose (GE Helthcare, Buckinghamshire, UK) to remove immunoglobulin of FBS in culture media.

The yield of the purified NC2 recombinant protein was about 0.8 mg/l of the 10% FBS supplemented medium. The purified NC2 recombinant protein was subjected to 15.0% SDS–PAGE analysis. The molecular weight of the NC2 protein was confirmed to be 34 kDa (Fig. 1B).

2.2.3. Preparation of recombinant NC2 domain produced by bacterial expression system

The NC2 DNA fragment was subcloned into the *Nco*I–*Xho*I site of the bacterial expression pET28a(+) vector (Novagen, Madison, WI). Then the 6 × His-Tag was added to the C-terminal.

The NC2 expression vector was transformed to *E. coli.* BL21(DE3) codon plus RIL (Stratagene, La Jolla, CA) by heat-shock methods. The recombinant NC2 was induced by standard protocols and was purified on Talon cobalt-chelate column according to the manufacturer's instructions. The yield of the purified recombinant NC2 was about 0.35 mg/l of the LB culture medium. The purified NC2 recombinant protein was subjected to 15.0% SDS–PAGE analysis. The analysis confirmed that the purified NC2 protein was 27 kDa (Fig. 1B). The difference of molecular weight between mammalian expressed NC2 and bacterial expressed NC2 may be due to the posttranslational modification.

2.3. Development of the ELISAs

2.3.1. NC1 ELISA (mammalian)

For NC1 ELISA, microtiter 96-well plates (Maxisorp; Nunc, Rochester, NY) were coated with purified recombinant NC1 at a concentration of 2 μg/ml in 20 mM carbonate buffer (pH 9.3) overnight at 4 °C. The coated plate was washed 2 times with PBS and blocked with PBS containing 1% bovine serum albumin (BSA) and 5% sucrose for 3 h at room temperature. In ELISA assays, sera from patients and normal donors were diluted 100-folds with PBS containing 0.15% Tween20, 1% casein enzymatic hydrolysate and 0.2 mg/ml *E. coli* extract. The diluted serum was added to the wells (100 μl/well). After incubation for 1 h at room temperature, the wells were washed 4 times with PBS containing 0.05% Tween20 (PBS-Tween20). Then, 100 μl of anti-human IgG conjugated to peroxidase (MBL, Nagoya, Japan) diluted 7000-fold in 20 mM HEPES (pH 7.4), 135 mM NaCl, 1% BSA and 0.1% hydroxyphenylacetic acid was added to each well. After incubation for 1 h at room temperature, plates were washed 4 times with PBS-Tween20, and bound antibodies were detected with 3,3′,5,5,′-tetramethylbenzidine as a substrate. After enzyme reaction for 30 min, the reaction solution was stopped by the addition of 100 μl/well of 1 N sulphuric acid. Plates were read at an optical density of 450 nm.

2.3.2. NC2 ELISA (mammalian)

For NC2 ELISA, 2 μg/ml of purified recombinant NC2 was coated onto microtiter 96-well plates at 4 °C overnight. NC2 ELISA was blocked and measured with the same method used for the NC1 ELISA.

2.3.3. NC1 (mammalian) + NC2 (bacterial) ELISA

For NC1 + NC2 ELISA, both purified recombinant NC1 produced by mammalian expression system and NC2 produced by bacterial expression system were coated onto microtiter 96-well plates at 4 °C overnight. Either a mixed solution of NC1 (2 μg/ml) and NC2 (1 μg/ml) or a mixed solution of NC1 (1 μg/ml) and NC2 (1 μg/ml) added to microtiter plates. NC1 + NC2 ELISA was measured the same method as NC1 ELISA.

2.4. Calculation of index

The index value was calculated by the following equation: (OD – negative control)/(positive control – negative control) × 100.

2.5. Immunoblot using the recombinant proteins and dermal extract

The recombinant proteins were size-fractionated by SDS–PAGE and transferred to a nitrocellulose membrane, and detected using a horseradish peroxidase-conjugated rabbit antihuman IgG antibody (Dako, Japan) in dilution 1:100. After washing of the strips three times in TBS washing buffer, revealing was done by HRP color development reagent (Bio-RAD). Dermal extract was run on 5% electrophoresis gel. Immunoblot was done by the same procedure.

2.6. IF

Indirect IF was carried out on salt-split skin using the patients' sera. The cryosection slides were washed in phosphate buffered saline for 5 min 3 times. Then incubated with blocking buffer (1% bovine serum albumin and 1 mM $CaCl_2$ in PBS) for 30 min at room temperature. The salt-split skin was incubated with the patients' sera dilution 1:40 in blocking buffer overnight at 4 °C. After washing with PBS 3 times, the binding was detected after incubation with polyclonal rabbit antihuman IgG FITC antibody (Dako, Japan) at the dilution of 1:100 for 1 h at room temperature.

2.7. Statistical analysis

Receiver-operating-characteristic (ROC) curves were analyzed to compare the performance of each assay. The area under the ROC curves was calculated using StatFlex software version 5.0 (Artech, Osaka, Japan).

3. Results

3.1. ELISA coated recombinant NC1 domain

To develop a sensitive and specific ELISA for detecting the autoantibodies against Type VII collagen in EBA patients, we first produced recombinant protein of NC1 which contains the entire

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1253 residues of the NC1 domain of type VII collagen by mammalian expression system. The purified recombinant NC1 domain produced by stably transfected CHO cells were immobilized on microtiter plates (Fig. 1B). We tested NC1 mammalian expression type VII collagen domain ELISA using EBA sera, normal control and disease control sera. The results are shown in Fig. 2A as a scatter plot. A cut off value was established as a value of 3SD above the mean of 55 normal sera. The mean was 3.06, resulting in a cut off value of 10.46. Only 1 NHS exceeded the cut off value. We then tested 49 EBA sera, 20 pemphigus vulgaris and 20 bullous pemphigoid sera as disease control sera. 45/49 EBA sera and 1 NHS exceeded the cut off value while none of the PV and BP sera exceeded the cut off value. This ELISA had 91.8% sensitivity and 98.1% specificity.

3.2. Development of ELISA coated with recombinant NC2 domain

We then sought to characterize EBA patients' sera using NC2 domain ELISA. First we used recombinant NC2 domain of type VII collagen produced by the mammalian expression system (Fig. 1B). We tested 49 EBA sera and 55 normal control sera. As shown in Fig. 2B, this ELISA had high background that led to non-specific binding of some normal human sera to the ELISA. This high background may be due to the low purity of the recombinant NC2 domain which was produced by CHO cells cultured in the medium containing FBS. The recombinant NC2 domain could not be produced by CHO cells in serum-free medium although the recombinant NC1 domain was prepared from CHO cells cultured in serum-free medium (data not shown).

Therefore, we tried to use recombinant NC2 domain of type VII collagen produced by bacterial expression system for this ELISA (Fig. 1B). As shown in Fig. 2C, the cut off value of the NC2 ELISA had a lower background. The mean for the bacterial expression NC2 ELISA was 2.23 with cut off value of 6.96. In this ELISA no NHS exceeded the cut off value. Eight EBA sera exceeded the cut off value. The ELISA using recombinant NC2 produced by the bacterial expression system could decrease the non-specific binding of normal control sera. In addition, the correlation coefficient between mammalian expression NC2 ELISA and bacterial expression NC2 ELISA by EBA sera was 0.9, indicating that the recombinant NC2 produced by bacterial expression retained epitopes bound by EBA sera.

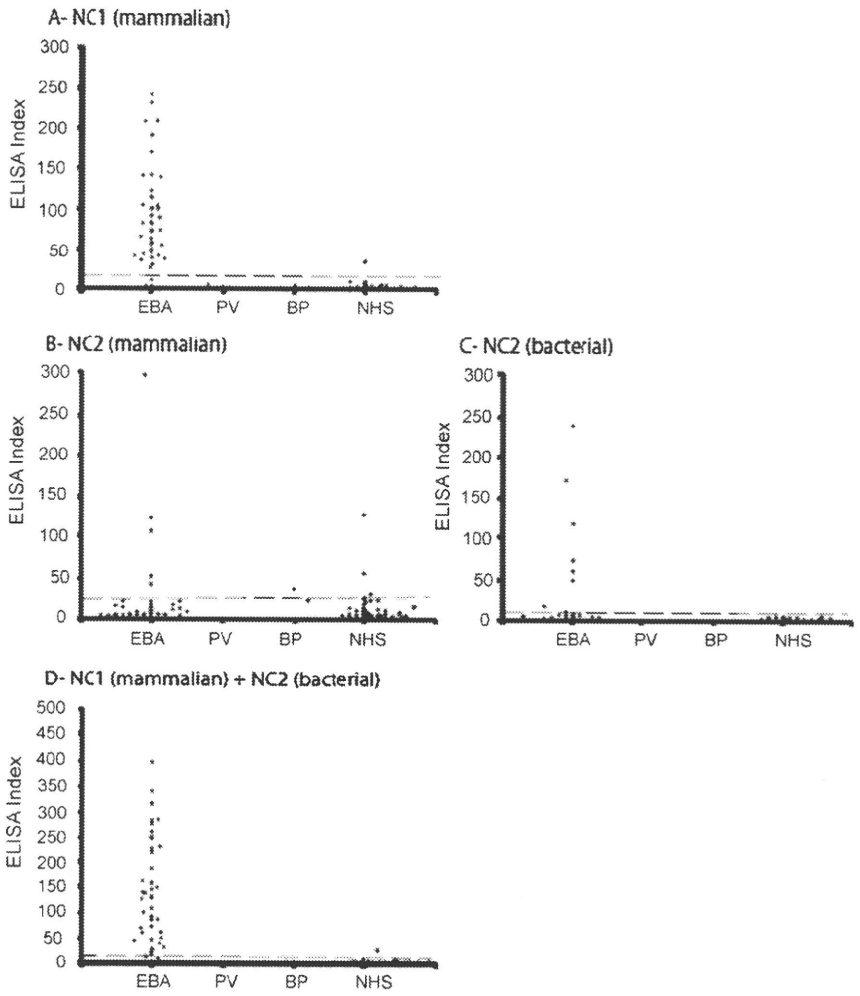

Fig. 2. Scatter plot representation of NC1, NC2, and NC1 + NC2 ELISAs. EBA, PV, BP and normal control (NHS) sera were incubated with ELISA immobilized indicated recombinant proteins and the bound antibodies were detected by peroxidase conjugated anti-human IgG antibody. The horizontal line indicates a cut off value, defined as average plus 3SD of normal control sera. (A) ELISA using recombinant NC1 domain produced by mammalian expression system; (B) ELISA using recombinant NC2 domain produced by mammalian expression system; (C) ELISA using recombinant NC2 produced by bacterial expression system; (D) ELISA using the combination of recombinant NC1 and recombinant NC2 domains. The ELISA plates were immobilized with purified 2:1 weight mixture of NC1 (mammalian) and NC2 (bacterial) recombinant proteins.

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Table 1
Summary of the antibody profiles of all tested sera by ELISAs using either recombinant NC1 or recombinant NC2.

NC1	NC2	Number ($n = 49$)	Percentage
+	+	7	14.2
+	–	38	77.5
–	+	1	2.0
–	–	3	6.1

3.3. Summary of antibody profile of EBA sera by NC1 ELISA and NC2 ELISA

Antibody profiles against NC1 and NC2 domains of tested EBA sera are shown in Table 1. Forty-six sera out of 49 sera were positive in either NC1 or NC2 ELISA. Seven sera reacted to both NC1 and NC2 ELISAs. 38 EBA sera reacted to only NC1 domain. One EBA serum reacted to only NC2 domain ELISA. Three sera were negative by both ELISAs although the sera were positive by salt-split IF.

3.4. Development of ELISA coated with a mixture of recombinant NC1 + NC2 domain

There was one EBA serum which bound to NC2 but did not bind to NC1 domain of type VII collagen. Therefore, to increase the sensitivity of the ELISA, we decided to combine the NC1 domain produced by mammalian expression and the NC2 domain produced by bacterial expression as substrates of the ELISA. We used a 2:1 ratio of the NC1 and NC2 ((2 μg/ml) and (1 μg/ml)). The mean of the ELISA was 4.47 with resultant cut off value of 10.94 (Fig. 2D). 46/49 EBA sera and 1 NHS exceeded the cut off value in both ELISAs. This ELISA had 93.8% sensitivity and 98.1% specificity.

We analyzed receiver-operating-characteristic (ROC) curves for NC1, NC2 and the combination of NC1 + NC2 ELISAs to compare the performance of each assay (Fig. 3). NC1 + NC2 domain ELISA had the greatest area under the ROC curves, which supports that NC1 + NC2 ELISA is a more accurate assay for the diagnosis of EBA than either NC1 or NC2 ELISAs.

3.5. Comparing the ELISA to other conventional assays for the diagnosis of EBA

Next, we compared the performance of the NC1 + NC2 ELISA with those of conventional methods; i.e., salt-split skin IF and immunoblot using dermal extracts (Table 2). Forty-six sera out of 49 sera (93.9%) were positive by the NC1 + NC2 ELISA while 44 sera (89.8%) were positive by immunoblot. Three sera were positive by ELISA but were negative by immunoblot. One serum was negative by ELISA but was positive by immunoblot. Two sera were negative by both ELISA and immunoblot. All sera were positive by salt-split skin IF. These data suggest that this NC1+ NC2 ELISA is comparable to or slightly more sensitive than immunoblot but salt-split skin IF is the most sensitive.

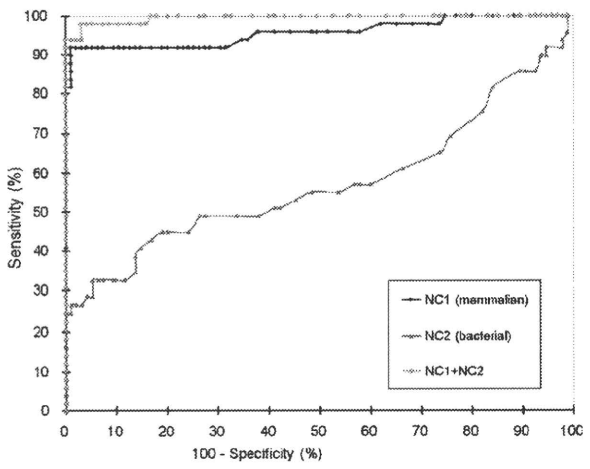

Fig. 3. Receiver operating characteristic curves comparing the performance of the NC1 and NC2 domains of type VII collagen ELISAs. NC1 + NC2 ELISA had the greatest area under the ROC curve.

Table 2
Comparison between NC1 + NC2 ELISA and immunoblot using dermal extracts.

		Immunoblot		Total
		+	–	
ELISA	+	43	3	46
	–	1	2	3
Total		44	5	49

3.6. Analysis for time course study

Finally, we analyzed whether the titer of the autoantibody measured by the NC1 + NC2 ELISA correlates with the disease activity of two EBA cases. We analyzed one case prospectively (Fig. 4A) and one case retrospectively (Fig. 4B) with this NC1 + NC2 ELISA.

Case A was a 25-year-old Japanese female with extensive blisters and erosions all over her body. She was initially treated by intravenous pulse steroid therapy followed by oral betamethasone 6 mg, azathioprine 25 mg/day and colchicines 1 mg/day. The clinical disease activity was assessed by pemphigus disease area index [18], because currently there is no scoring system for EBA. As shown in Fig. 4A, the disease activity subsided gradually along with the decreasing ELISA titers. We used the ELISA titer as a guide to taper the steroids and to increase the dose of azathioprine to 40 mg to control the disease.

Case B was a 49-year-old Japanese female with oral erosions and scattered skin blisters and erosions localized on her extremities. The patient was initially treated with prednisolone 10 mg/day without apparent improvement as mechanical blisters were observed on her extremities. After adding minocycline 100 mg on the 5th month, the cutaneous lesions gradually cleared. The ELISA titer decreased along with the clinical disease activity. The ELISA indexes tend to fluctuate in parallel with the disease activities in the two cases.

4. Discussion

EBA is considered a challenging disease both in the diagnosis and in the treatment. In this study, we tried to develop a sensitive and specific ELISA to detect anti-type VII collagen autoantibodies in EBA patients for practical use. We developed 3 ELISAs: NC1 domain (mammalian), NC2 domain (mammalian or bacterial), combination of NC1(mammalian) + NC2 (bacterial). We evaluated them with 49 EBA sera, disease control sera and normal control sera. We found that combination of NC1 (mammalian) + NC2 (bacterial) domain ELISA had the highest sensitivity and specificity (93.8% sensitivity and 98.1% specificity). Therefore, we chose the combination of NC1 + NC2 as substrates of the ELISA for practical usage.

ELISAs for EBA have been developed previously [9,17]. Chen et al. reported ELISA using recombinant NC1 domain of type VII collagen produced by eukaryotic expression system similar to our recombinant NC1 protein. They reported that all 24 tested EBA sera reacted with the NC1 by ELISA and none of controls reacted with the NC1. The difference between our and their

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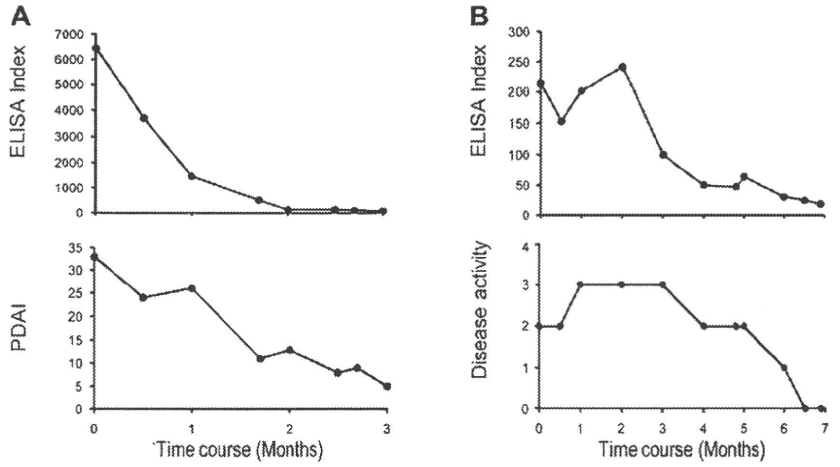

Fig. 4. Time course of two EBA patients. NC1 + NC2 ELISA index fluctuates in parallel with the disease activity in two EBA cases. Severe = 3, moderate = 2, mild = 1, clinically free = 0. For case A, pemphigus disease area index (PDAI) was used to show the disease activity [19].

ELISA is that we used the combination of NC1 domain and NC2 domain as substrates. According to our results, 2% of the tested EBA patients bound only to the NC2 domain. As EBA is a very rare disease, we believe that it is important not to miss the EBA patients who possess autoantibodies against only the NC2 domain.

The rationale for NC1 + NC2 ELISA is to use the recombinant protein produced by same expression system for NC1 and NC2. However, as we could not produce the NC2 recombinant protein in serum-free medium for unknown reason, we produced the recombinant NC2 protein by CHO cells cultured in FBS-containing medium. The difficulty of purification of the NC2 recombinant protein (mammalian) caused the high background of mammalian NC2 ELISA. Therefore, we chose the bacterial expression system for recombinant NC2 domain. Meanwhile, we did not use bacterial expression system for recombinant NC1 domain because we speculated that the recombinant protein may be easily degraded due to its large molecular size.

We compared the performance of the ELISA with conventional assays such as immunoblot using dermal extract and indirect IF using salt-split skin. It seems that the sensitivity of the ELISA is slightly higher than that of immunoblot. Three EBA sera were positive by ELISA but were negative by immunoblot. It is known that a portion of the NC2 domain is proteolytically removed during the procollagen to collagen conversion in tissues. Therefore, there may be a possibility that the sera recognize a small part of the NC2 that was cleaved out [6,18]. However, the three sera did not react with the NC2 by ELISA, suggesting that the difference of the reactivities of the NC1 + NC2 ELISA and the immunoblot with dermal extract may be due to the difference of the sensitivities of the assays, not due to the epitope binding. The higher sensitivity in ELISA may be due to the fact that recombinant proteins in ELISA retain native conformation of type VII collagen whereas the recombinant antigen used for immunoblot is denatured protein. Alternatively, this may also reflect that the substrate for the IB is dermal extract which contains mixture of various antigens. Salt-split skin IF is still more sensitive to diagnose EBA than the ELISA and the immunoblot. However, taking into consideration that the technique is not routinely done in many institutions, the ELISA can be considered a practical assay. It should be noted that the final diagnosis cannot be made only by the salt-split IF in some cases.

Two sera were negative by both ELISA and immunoblot using dermal extract. The criteria used for the diagnosis of EBA is typical clinical and histological features of EBA, and the immunoreactivity to the dermal side of the dermal–epidermal junction by salt-split skin. Therefore, we cannot exclude the possibility that the cases have other bullous disease that can react with the dermal side by salt-split IF such as anti-laminin γ1 pemphigoid. However, as the sera did not show 200 kDa protein binding by immunoblot using dermal extract, the cases may not have anti-laminin γ1 pemphigoid.

One case was negative by ELISA but was positive by immunoblot using dermal extract. It has been reported that autoantibodies in some EBA patients bind only to the central collagenous domain of type VII collagen [14]. The serum of this case may bind to the central collagenous domain of type VII collagen. We could not include the whole type VII collagen as a substrate of the ELISA because of the difficulty of production of the high-molecular-weight protein.

We examined whether there is any correlation between the epitope profile of autoantibodies and the clinical features of the EBA patients including classical non-inflammatory type vs inflammatory type. We compared two cases which had antibodies against only NC1 domain, one case which had antibodies against only NC2 domain and two cases which had antibodies against both NC1 and NC2 domains. The distribution of the disease, inflammation and scarring were nearly the same. Oral mucosal affection, and nail dystrophy were observed in all. We could not find correlation between the clinical features and the antibody profile against NC1 and NC2 domains. This coincided with the previous reports that no correlation was found between the clinical features and the epitopes [11].

Another advantage of ELISA is that it can quantify the autoantibody titers. It was not previously clear whether antibody titers correlate with the disease activity in EBA. In Fig. 4, case A and B showed that the autoantibodies measured by the ELISA clearly fluctuated in parallel with the disease activity. These findings suggest that ELISA may be valuable for monitoring the disease activity in some cases. ELISA scores may be useful to determine the schedules of tapering corticosteroids and to choose therapeutic plans. To clarify this issue, studies using more cases will be needed in the future.

In summary, we have established an ELISA system using the combination of NC1 and NC2 domains of type VII collagen to detect autoantibodies against type VII collagen for EBA. This ELISA system will be useful in clinical practice to make proper diagnosis and evaluate the disease activity for EBA.

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Conflict of interest

Ms Kim and Dr. Murakami belong to Medical and Biological Laboratories Co. Ltd. who supply anti-type VII collagen ELISA kit described in this study.

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References

[1] Woodley DT, Briggaman RA, Okeefe EJ, Inman AO, Queen LL, Gammon WR. Identification of the skin basement-membrane auto-antigen in epidermolysis bullosa-acquisita. N Engl J Med 1984;310:1007–13.
[2] Woodley DT, O'Keefe EJ, Reese MJ, Mechanic GL, Briggaman RA, Gammon WR. Epidermolysis bullosa acquisita antigen, a new major component of cutaneous basement membrane, is a glycoprotein with collagenous domains. J Invest Dermatol 1986;86:668–72.
[3] Woodley DT, Chang C, Saadat P, Ram R, Liu Z, Chen M. Evidence that anti-type VII collagen antibodies are pathogenic and responsible for the clinical, histological, and immunological features of epidermolysis bullosa acquisita. J Invest Dermatol 2005;124:958–64.
[4] Woodley DT, Burgeson RE, Lunstrum G, Bruckner-Tuderman L, Reese MJ, Briggaman RA. Epidermolysis bullosa acquisita antigen is the globular carboxyl terminus of type VII procollagen. J Clin Invest 1988;81:683–7.
[5] Remington J, Chen M, Burnett J, Woodley DT. Autoimmunity to type VII collagen: epidermolysis bullosa acquisita. Curr Dir Autoimmun 2008;10: 195–205.
[6] Burgeson RE. Type VII collagen, anchoring fibrils, and epidermolysis bullosa. J Invest Dermatol 1993;101:252–5.
[7] Keene DR, Sakai LY, Lunstrum GP, Morris NP, Burgeson RE. Type VII collagen forms an extended network of anchoring fibrils. J Cell Biol 1987;104:611–21.
[8] Sakai LY, Keene DR, Morris NP, Burgeson RE. Type VII collagen is a major structural component of anchoring fibrils. J Cell Biol 1986;103:1577–86.
[9] Chen M, Doostan A, Bandyopadhyay P, Remington J, Wang X, Hou Y, et al. The cartilage matrix protein subdomain of type VII collagen is pathogenic for epidermolysis bullosa acquisita. Am J Pathol 2007;170:2009–18.
[10] Gammon WR, Murrell DF, Jenison MW, Padilla KM, Prisayanh PS, Jones DA, et al. Autoantibodies to type VII collagen recognize epitopes in a fibronectin-like region of the noncollagenous (NC1) domain. J Invest Dermatol 1993;100:618–22.
[11] Ishii N, Yoshida M, Hisamatsu Y, Ishida-Yamamoto A, Nakane H, Iizuka H, et al. Epidermolysis bullosa acquisita sera react with distinct epitopes on the NC1 and NC2 domains of type VII collagen: study using immunoblotting of domain-specific recombinant proteins and postembedding immunoelectron microscopy. Br J Dermatol 2004;150:843–51.
[12] Chen M, Keene DR, Costa FK, Tahk SH, Woodley DT. The carboxyl terminus of type VII collagen mediates antiparallel dimer formation and constitutes a new antigenic epitope for epidermolysis bullosa acquisita autoantibodies. J Biol Chem 2001;276:21649–55.
[13] Mayuzumi M, Akiyama M, Nishie W, Ukae S, Abe M, Sawamura D, et al. Childhood epidermolysis bullosa acquisita with autoantibodies against the noncollagenous 1 and 2 domains of type VII collagen: case report and review of the literature. Br J Dermatol 2006;155:1048–52.
[14] Ishii N, Yoshida M, Ishida-Yamamoto A, Fritsch A, Elfert S, Bruckner-Tuderman L, et al. Some epidermolysis bullosa acquisita sera react with epitopes within the triple-helical collagenous domain as indicated by immunoelectron microscopy. Br J Dermatol 2009;160:1090–3.
[15] Fukumoto T, Umekawa T, Higuchi M, Hashimoto T, Shumann H, Bruckner-Tuderman L, et al. Childhood epidermolysis bullosa acquisita with autoantibodies against all 3 structural domains of type VII collagen. J Am Acad Dermatol 2004;50:480–2.
[16] Gammon WR, Kowalewski C, Chorzelski TP, Kumar V, Briggaman RA, Beutner EH. Direct immunofluorescence studies of sodium chloride-separated skin in the differential diagnosis of bullous pemphigoid and epidermolysis bullosa acquisita. J Am Acad Dermatol 1990;22:664–70.
[17] Chen M, Chan LS, Cai X, O'Toole EA, Sample JC, Woodley DT. Development of an ELISA for rapid detection of anti-type VII collagen autoantibodies in epidermolysis bullosa acquisita. J Invest Dermatol 1997;108:68–72.
[18] Bruckner-Tuderman L, Nilssen O, Zimmermann DR, Dours-Zimmermann MT, Kalinke DU, Gedde-Dahl Jr T, et al. Immunohistochemical and mutation analyses demonstrate that procollagen VII is processed to collagen VII through removal of the NC-2 domain. J Cell Biol 1995;131:551–9.
[19] Rosenbach M, Murrell DF, Bystryn JC, Dulay S, Dick S, Fakharzadeh S, et al. Reliability and convergent validity of two outcome instruments for pemphigus. J Invest Dermatol 2009;129:2404–10.

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