



Validation of chemiluminescent enzyme immunoassay in detection of autoantibodies in pemphigus and pemphigoid



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ABSTRACT

Background: A novel chemiluminescent enzyme immunoassay (CLEIA) was recently developed to quantify autoantibodies specific for desmogleins (Dsgs) and BP180, the target antigens of pemphigus and pemphigoid. This assay is automated and highly accurate and efficient.

Objective: To validate the use of the CLEIA for detection of autoantibodies during the clinical courses of patients with pemphigus and pemphigoid.

Methods: To define cut-off values for Dsg1, Dsg3, and BP180, we evaluated 47 serum samples from patients with pemphigus foliaceus (PF), 59 from those with pemphigus vulgaris (PV), 52 from those with bullous pemphigoid (BP), and 995 from healthy individuals. We also evaluated any fluctuations in CLEIA titers according to disease activity during the clinical course of 10 cases each of PF, PV, and BP. We used clinical symptom scores, the pemphigus disease area index (PDAI) and the bullous pemphigoid disease area index (BPDAI), to evaluate disease activity.

Results: The cut-off values for the CLEIA titers determined by the Youden index were 15.4 U/mL for Dsg1, 14.9 U/mL for Dsg3, and 16.8 U/mL for BP180. CLEIA titers fluctuated in parallel with the PDAI/BPDAI scores in 28 of the 30 cases with PF, PV, or BP. Although the CLEIA and enzyme-linked immunosorbent assay (ELISA) values in the same samples differed substantially in some cases, the concordance rates of positive/negative results between the CLEIA and ELISA were 96% for Dsg1, 97% for Dsg3, and 96% for BP180.

Conclusion: The CLEIA, a newly developed, highly effective autoantibody detection system, is as reliable as ELISA for evaluation of the clinical courses of pemphigus and pemphigoid.

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1. Introduction

Autoimmune bullous diseases are caused by autoantibodies that react with adhesion molecules in the skin. Because sequential identification of disease-specific target antigens is important, detection of autoantibodies targeting specific molecules is one of the most important steps in the diagnosis of autoimmune

blistering diseases. In particular, enzyme-linked immunosorbent assays (ELISA) using recombinant desmogleins (Dsgs) and BP180 (BPAG2, collagen XVII) have been widely used for serum analyses in patients with pemphigus and pemphigoid [1–4]. Pemphigus is characterized by intraepithelial blister formation attributed to IgG autoantibodies specific for Dsgs, which are protein components of the desmosomes that play crucial roles in cell–cell adhesion in the epidermis. Because ELISA can distinguish between Dsg1 and Dsg3, these assays are used to detect the two major classic forms of pemphigus, pemphigus vulgaris (PV) and pemphigus foliaceus (PF) [2,5–8]. The clinical phenotypes of pemphigus are generally explained by the Dsg compensation theory of autoantibody profiles. Thus, patients with PF express anti-Dsg1 autoantibodies

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only, those with the mucous-dominant type of PV express anti-Dsg3 autoantibodies only, and those with the mucocutaneous type of PV express both anti-Dsg3 and anti-Dsg1 autoantibodies [2,5,9]. Another major type of autoimmune bullous disease is pemphigoid, characterized by subepidermal blisters caused by IgG autoantibodies targeting essential protein components of hemidesmosomes involved in dermal-epidermal cohesion. Detection of anti-BP180 autoantibodies by ELISA is helpful in the diagnosis of bullous pemphigoid (BP), which affects most patients with pemphigoid [2–5,9–12].

In patients with PV, PF, and BP, the sensitivity and specificity of ELISA for the detection of relevant circulating autoantibodies have been confirmed. In addition, because ELISA titers (ELISA index values) correlate with disease activities [1,3,4,6,8,13–17], they are useful for the assessment of disease activity after successful treatment of blisters and erosions. However, we have encountered some cases of autoimmune blistering diseases associated with high autoantibody titers that did not show an evident decrease in ELISA indices after clinical improvement [13]. In such cases, to obtain reliable index values, ELISA must be performed using appropriate serum dilutions to demonstrate linear correlations between the dilutions and index values. These are the so-called “true” index values, enabling comparison of autoantibody titers between the active and remission stages. As ELISA is based on an enzyme reaction, sera with high-titer antibodies (typically with an ELISA index >100) can plateau or reach saturation, thus yielding index values that are lower than the actual values. In other words, the antigen-antibody reaction becomes saturated due to excess autoantibodies [13,18].

The chemiluminescent enzyme immunoassay (CLEIA) is a newly developed immunoassay based on light emission, and it is generally more efficient and sensitive than ELISA. Using magnetic bead-bound antigen particles, the CLEIA can be applied to detect autoantibodies targeting Dsgs and BP180. The entire procedure is fully automated using the STACIA[®] system. The CLEIA has previously been used to measure blood drug concentrations, isozyme levels, and components of the coagulation system [19,20]. The CLEIA is efficient, reducing the time of measurement of autoantibodies in patients with pemphigus and pemphigoid to 20 min compared with approximately 3 h for ELISA. Because CLEIA uses a photomultiplier tube with a highly sensitive photodetector, the dynamic range of detection is wider than that of ELISA, and unlike ELISA, it is not necessary to dilute high-titer sera to obtain a “true” index values; valid autoantibody levels can be up to 1000 U/mL [19].

In the present study, we explored the characteristics of CLEIA detection of autoantibodies in patients with PV, PF, and BP. First, we established cut-off values by comparing data from affected versus normal individuals. We next compared the CLEIA indices and the clinical severity scores of patients with pemphigus and pemphigoid. We also compared the CLEIA and ELISA indices to determine an intercorrelation.

Table 1
Demographics of the patients used in this study.

Diagnosis	PV	PF	BP
patients	60	47	52
samples	115	102	103
Age(average)	12–93 (54.7)	18–87 (58.3)	0–90 (66.7)
Sex(M/F)	21/39	22/25	20/32

PV: pemphigus vulgaris, PF: Pemphigus foliaceus, BP: Bullous pemphigoid.

2. Materials and methods

2.1. Subjects and serum samples

All serum samples from patients with autoimmune bullous diseases evaluated in this study were collected in the Department of Dermatology of Keio University between 2001 and 2013. A total of 115 serum samples from 60 PV patients, 102 from 47 PF patients, and 103 from 52 BP patients were obtained (Table 1). All patients were diagnosed based on typical clinical features and histopathological and immunological findings. Sera were obtained from patients exhibiting different levels of disease activity (i.e., in the active or remission stage). By referencing clinical symptoms and antibody profiles, the PV cases were classified as the mucosal-dominant or mucocutaneous type. To explore the correlations between CLEIA and ELISA scores and clinical disease activity, serial serum samples from 10 cases each of PV, PF, and BP were collected.

This study was approved by the institutional review board of Keio University and was conducted in line with the principles of the Declaration of Helsinki. All samples were used after obtaining informed consent. We used 995 commercially available serum samples from healthy individuals living in the United States (SLR Research Corporation, Carlsbad, CA, USA) to determine CLEIA cut-off values.

2.2. ELISAs for Dsg1, Dsg3, and BP180

Antibody titers against Dsg1, Dsg3, and BP180 were measured using commercially available ELISA kits. For Dsg1 and Dsg3, we employed the MESACUP-2 DSG1/DSG3 ELISA kits (Medical & Biological Laboratories Co. Ltd; MBL, Nagoya, Japan), which use recombinant extracellular domains of Dsg1 and Dsg3 produced by CHO cells. For BP180, we used the MESACUP BP180 ELISA kit (MBL), which employs a recombinant NC16a domain of BP180 produced as a fusion protein in *Escherichia coli*. The ELISA index of each sample was evaluated as reported previously [2]. An ELISA index value >20.0 was considered positive for Dsg1 and Dsg3, and a value >9.0 was considered positive for BP180 [5]. All serum samples were initially diluted 1:100, and those samples with index values >100 were serially diluted up to 1:25,600 as appropriate (i.e., to achieve an ELISA index of 50–100).

2.3. CLEIAs for Dsg1, Dsg3, and BP180

Antibody titers against Dsg1, Dsg3 and BP180 were measured using the new CLEIA method, which is fully automated by integration with the STACIA[®] system (LSI Medience Corporation, Tokyo, Japan). Each recombinant protein for CLEIA was prepared as same as ELISA, i.e. recombinant extracellular domains of Dsgs produced by CHO cells and a recombinant NC16a domain of BP180 produced as a fusion protein in *E. coli*.

Serum samples were reacted with magnetic beads coated with recombinant Dsg1, Dsg3, or BP180 proteins (MBL). Next, the immunocomplexes were reacted with alkaline phosphatase-labeled IgG polyclonal antibody. After the substrate liquid (CDP-STAR) was added, a chemiluminescent reaction commenced. A photomultiplier was used as the detector, which increased the dynamic range. Only sera with index values >1000 U/mL require dilution. This can be done automatically, but we performed manual dilutions to conserve the serum samples.

2.4. Assessment of disease activity

Clinical disease activity was assessed regularly using the pemphigus disease area index (PDAI) in patients with PV and PF [21–23] and the bullous pemphigoid disease area index (BPDAI) in

patients with BP [24,25], as described previously. The PDAI, which ranges from 0 to 263, assigns scores to defined anatomical regions based on the number and size of lesions. The PDAI is used to assess disease severity as follows: ≤ 8 , mild; 9–24, moderate; and ≥ 25 ,

severe [22]. The BPDAI differs from the PDAI in that the scores are calculated by counting the numbers of “erosion/blisters” and incidences of “urticaria/erythema”. The highest score is 360; no consensus on the assessment of mild/moderate/severe disease activity by the BPDAI has yet been attained [24]. The damage score of BPDAI is not included in this study. The PDAIs and BPDAs of several patients were re-scored retrospectively based on photographs and descriptions in medical records.

2.5. Statistical analysis

All statistical analyses were performed using Stat Flex ver. 6. Receiver operating characteristic curve analysis was performed, and cut-off CLEIA indices for Dsg1, Dsg3, and BP180 were determined using the Youden index.

3. Results

3.1. Establishing cut-off values for antibodies specific to Dsg1, Dsg3, and BP180 measured by the CLEIA

To determine cut-off values for autoantibodies measured using the CLEIA, we analyzed 46 PF serum samples for Dsg1, 59 PV sera for Dsg3, and 52 BP sera for BP180. All samples were positive for each antigen according to ELISA. As controls, 995 commercially available sera from healthy individuals were used. In addition, sera from patients with PF and PV served as controls for the BP180 CLEIA, and sera from patients with BP served as controls for the Dsg1 and Dsg3 CLEIAs. The Youden index (the maximum value of {sensitivity + specificity – 1}) was used to determine cut-off values. As shown in Fig. 1, the cut-off value for Dsg1 was 15.4 U/mL (sensitivity = 1, specificity = 1), that for Dsg3 was 14.9 U/mL (sensitivity = 1, specificity = 1), and that for BP180 was 16.8 U/mL (sensitivity = 0.96, specificity = 0.99).

To determine whether these cut-off values were consistent with the clinical data, we analyzed the distributions of the CLEIA scores for Dsg1, Dsg3, and BP180 in patients with PF, PV, and BP as well as in normal individuals (Fig. 2). Two serum samples from normal individuals yielded values very close to the cut-off values; one yielded a BP180 level of 16.8 U/mL and the other a Dsg1 level of 15.4 U/mL. Two serum samples from patients with PF had titers above the Dsg3 cut-off value (138.7 and 92.3 U/mL), and one serum sample from a PF patient was considered positive for BP180 (29.2 U/mL).

3.2. Correlations between CLEIA index values and disease activity

The correlations between disease activity (PDAI scores for PF and PV and BPDAI scores for BP) and the CLEIA values were examined over time in 10 cases each of PF, PV (four mucosal-dominant and six mucocutaneous cases), and BP. The time courses of the typical cases are shown in Fig. 3. As ELISA have traditionally been used to detect autoantibodies specific to Dsgs and BP180, we compared CLEIA and ELISA values for each case.

In 9/10 PF cases, 9/10 PV cases, and 10/10 BP cases, the antibody titers measured by both CLEIA and ELISA tended to show consistent fluctuations with disease activity in terms of the PDAI or BPDAI (Fig. 3, Supplementary Fig. 1). In one PF case (Fig. 3B), neither the CLEIA nor ELISA titer decreased sharply, even though the PDAI had improved after the initial treatment. In one case with PV (Fig. 3H), the Dsg1 titers of both the CLEIA and ELISA increased, even though the PDAI had decreased at the beginning of the disease course. In fact, in this case, the CLEIA Dsg3 titer changed in parallel with the PDAI, whereas the ELISA Dsg3 index increased slightly. With the exception of these two cases, changes in the CLEIA values reflected changes in disease activity, as did

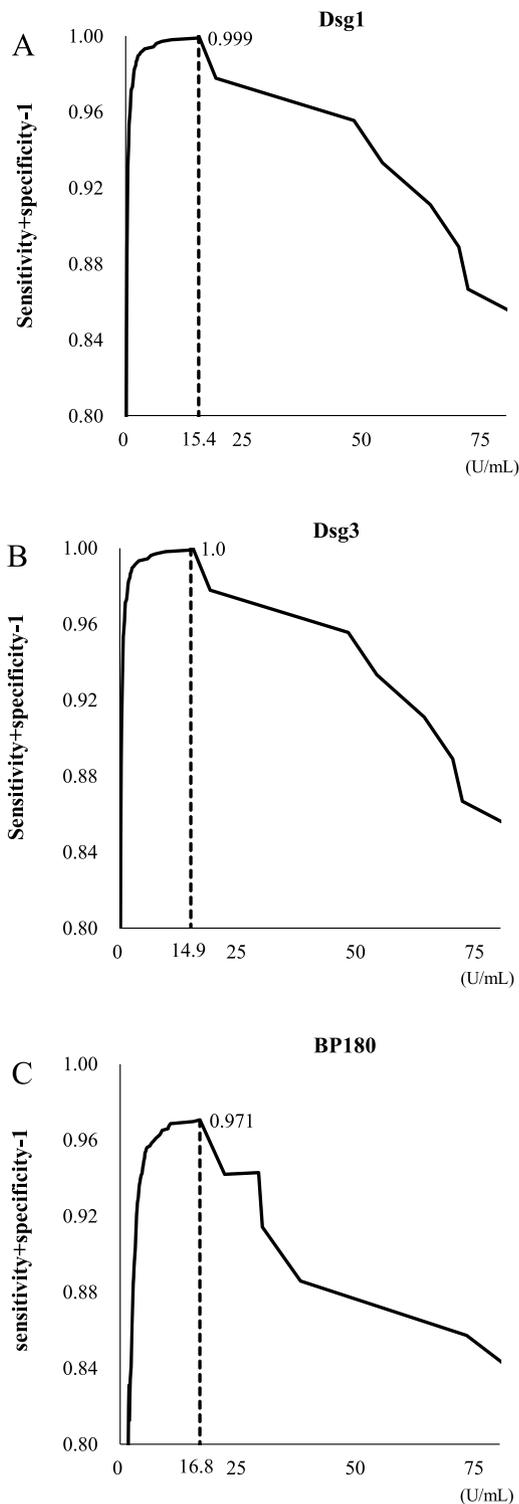


Fig. 1. Youden index values used to determine the CLEIA index cut-offs for Dsg1 (A), Dsg3 (B), and BP180 (C). The maximum sums of sensitivity and specificity were 15.4 for Dsg1, 14.9 for Dsg3, and 16.8 for BP. CLEIA: chemiluminescent enzyme immunoassay, Dsg: desmoglein, BP: bullous pemphigoid.

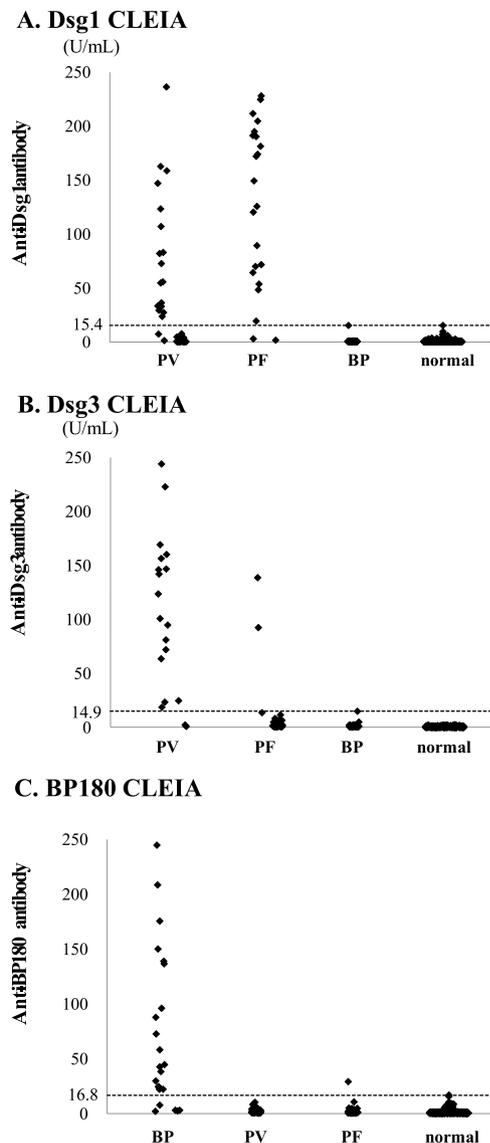


Fig. 2. Antibody titers against Dsg1, Dsg3, and BP180 measured by the CLEIA. The distributions of the anti-Dsg1 (A), anti-Dsg3 (B), and anti-BP180 (C) titers obtained in the CLEIA are shown.

PV: pemphigus vulgaris, PF: pemphigus foliaceus, BP: bullous pemphigoid. CLEIA: chemiluminescent enzyme immunoassay.

changes in the ELISA values. Even in the exceptional cases mentioned above, the CLEIA titers fluctuated in parallel with disease activity during the later stages. The absolute CLEIA values at the times at which the highest PDAI/BPDAI scores were observed tended to be higher than the absolute ELISA values [in 9/10 cases for BP180, in 8/10 cases for Dsg3, and in 13/20 cases (6/10 PF and 7/10 PV) for Dsg1].

3.3. Correlations between CLEIA and ELISA index values

We found that the CLEIA titers generally fluctuated in parallel with disease activity in all cases of PF, PV, and BP. Because ELISA is a well-established method, dermatologists may find it necessary to compare CLEIA and ELISA index values throughout the clinical course of patients with autoimmune blistering diseases. We explored the correlations between ELISA and CLEIA values for each antigen. From representative results, it revealed that CLEIA had a

wider dynamic range of testing sera than ELISA in each antigen (Fig. 4). While CLEIA could detect up to 800 U/mL value of antibodies without dilution, ELISA indexes reached plateau between 100 and 150 for each antigen (Dsg1, Dsg3, and BP180). As previously mentioned, samples with index values ≥ 100 should be serially diluted as appropriate in ELISA. For Dsg1, the maximum, minimum, and median indices for ELISA were 23,656.8, 0.8, and 99.1, and those for the CLEIA were 34,640.0, 0.1, and 136.3, respectively. For Dsg3, the maximum, minimum, and median indices for ELISA were 8,903.8, 0.1, and 70.8, whereas those for the CLEIA were 22,030.0, 0.4, and 112.1, respectively. For BP180, the maximum, minimum, and median indices for ELISA were 5,815.5, 0.6, and 74.1, and those for the CLEIA were 38,270, 1.9, and 347.8, respectively.

In general, the ELISA and CLEIA indices were correlated, with coefficients (r^2 values) of 0.80 for Dsg1, 0.81 for Dsg3, and 0.85 for BP180 (Fig. 5). Unfortunately, we could not estimate CLEIA values from ELISA data in the same samples and thus could not predict whether the CLEIA or ELISA value was higher in any given sample. The proportions of samples with higher CLEIA indices were 48% for Dsg1, 65% for Dsg3, and 86% for BP180; the CLEIA/ELISA index ratios were 1.10 for Dsg1, 1.67 for Dsg3, and 3.75 for BP180 (Table 2). Samples with ratios of 0.5–2.0 accounted for 60% of the Dsg1 samples, 56% of the Dsg3 samples, and 34% of the BP180 samples, suggesting that the difference in antibody titers between CLEIA and ELISA might be greater for BP180 than for Dsg1 or Dsg3. When the ratio range was increased to 0.2–5.0, samples within this range accounted for 79% of the Dsg1 samples, 95% of the Dsg3 samples, and 76% of the BP180 samples. Thus, to include over 90% of the samples for all antigens, the CLEIA/ELISA ratio range had to be increased to 0.1–10.0 (Table 2).

3.4. Concordance (positive or negative) between the CLEIA and ELISA data

As shown above, in some cases, the ELISA and CLEIA index values differed appreciably even within the same sample; thus, estimating one value from the other was impractical. We next examined whether the positive or negative results for an antigen were consistent between the two methods. In terms of Dsg1 and Dsg3, we evaluated 105 serum samples collected at different time points (i.e., in the active and remission stages) from PF and PV patients.

In the Dsg1 CLEIA, 73 serum samples exceeded the cut-off value (positive), and 32 were negative, whereas in the Dsg1 ELISA, 75 samples were positive and 30 negative (Table 3A); therefore, the agreement between the positive and negative CLEIA and ELISA results was 98%. In two samples determined to negative by the CLEIA but positive by ELISA, the CLEIA titers were 1.3 U/mL and 7.3 U/mL, and the ELISA index values were 26.8 and 40.6. The former case was in remission and the latter in the active stage of PF.

In the Dsg3 CLEIA, 59 samples were positive and 46 negative, whereas in the Dsg3 ELISA, 60 samples were positive and 45 negative (Table 3B), with an agreement of 99% between the positive and negative CLEIA and ELISA results. In the patient with a negative CLEIA but positive ELISA result, the CLEIA value was 13.6 U/mL, and the ELISA index was 26.7; this was a PF case with active disease exhibiting a high anti-Dsg1 antibody titer (23,656.8 by ELISA and 34,640.0 U/mL by CLEIA).

For BP180, we evaluated 52 serum samples collected from BP patients at different time points (i.e., in the active and remission stages). According to the CLEIA, 47 samples were positive and 5 negative for BP180, whereas according to ELISA, 49 were positive and 3 negative (Table 3C), with an agreement between the positive and negative CLEIA and ELISA results of 96%. Two samples differed in terms of positivity/negativity for BP180 between the CLEIA and

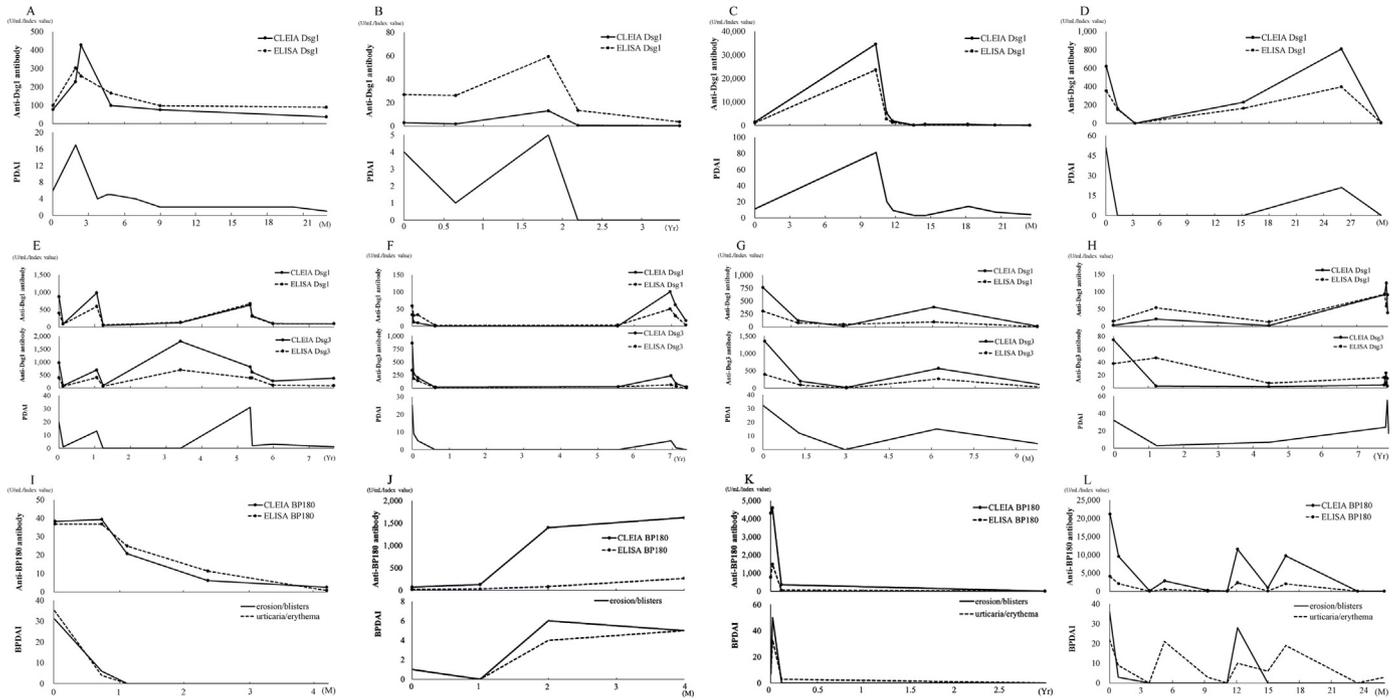


Fig. 3. CLEIA scores tended to fluctuate in parallel with disease activity over time. PF patients (A–D), PV patients (E and H: mucosal type; F and G: mucocutaneous type), and BP patients (I–L) were analyzed. In cases B and H, neither the CLEIA nor ELISA scores fluctuated in synchrony with the clinical manifestations during the first period evaluated. The other patients exhibited good correlations between the PDAI and BPDAl scores. CLEIA: chemiluminescent enzyme immunoassay, ELISA: enzyme-linked immunosorbent assay, PF: pemphigus foliaceus, PV: pemphigus vulgaris, BP: bullous pemphigoid. PDAI: pemphigus disease area index, BPDAl: bullous pemphigoid disease area index.

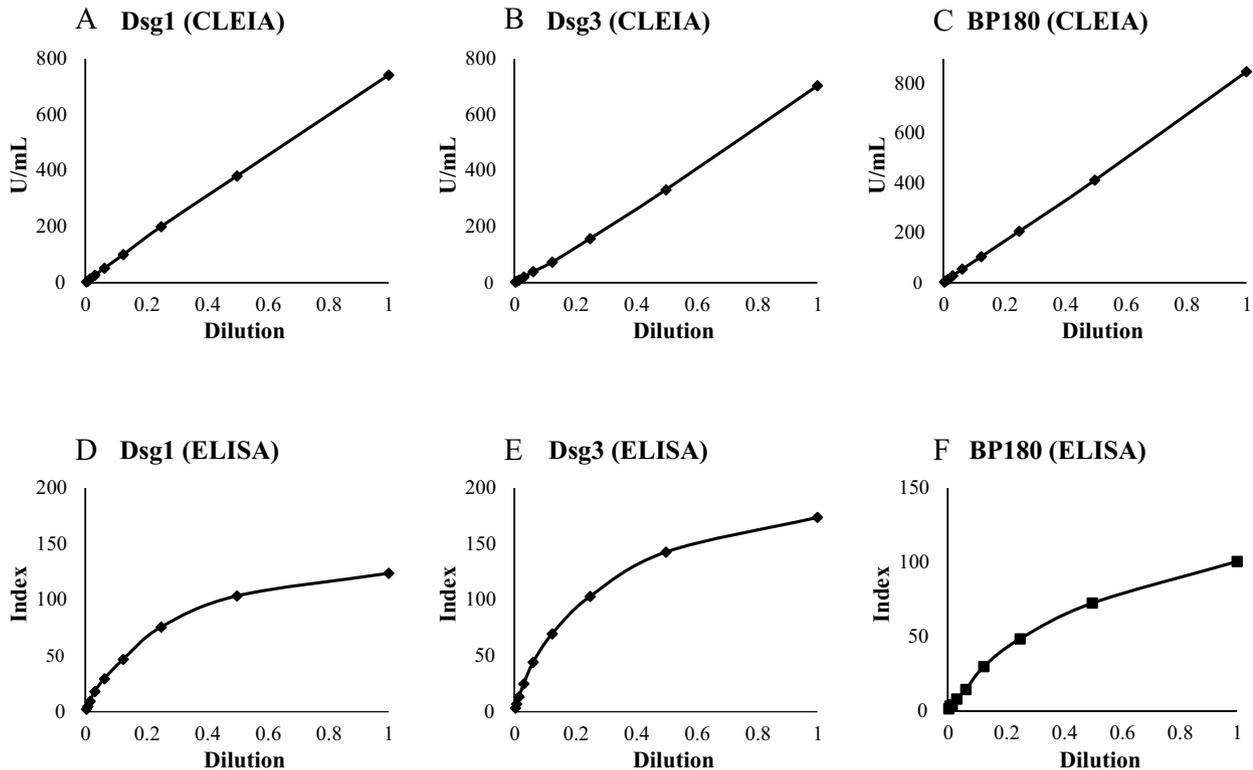


Fig. 4. Difference of dynamic ranges between CLEIA and ELISA index values. CLEIA could keep a positive linear relation between the dilution and antibody titers of sera (A–C). ELISA showed a plateau reaction at dilutions of 1.0–0.5, suggesting more dilution was necessary under situations with concentrated antibodies (D–F).

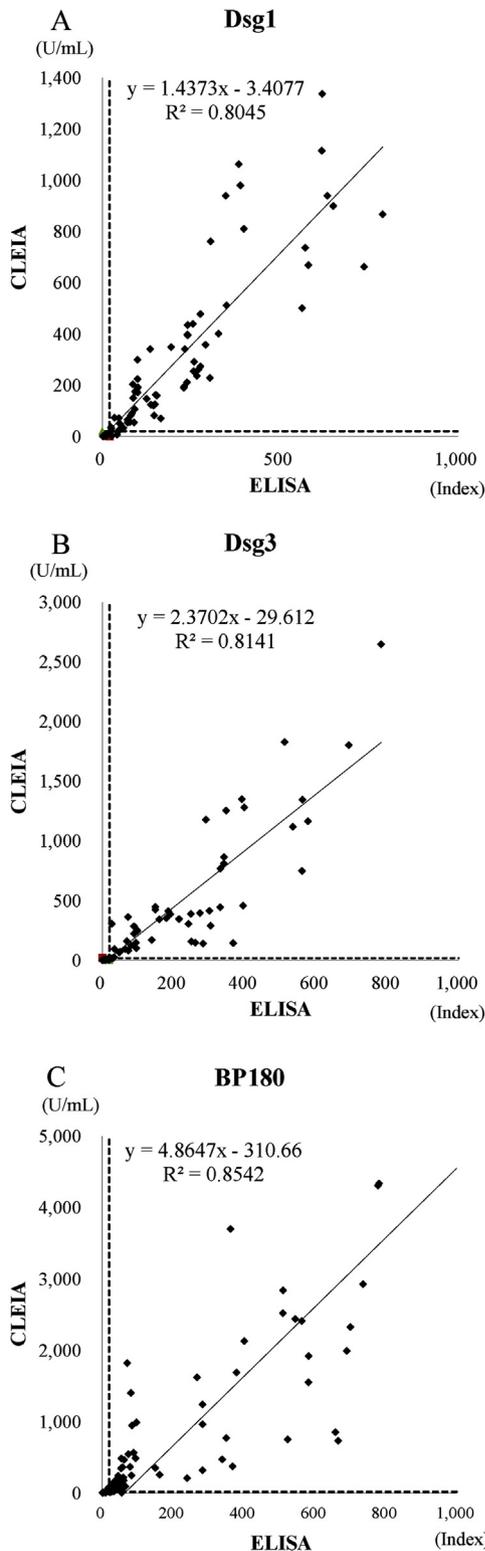


Fig. 5. Correlations between CLEIA and ELISA indices. The correlations between CLEIA and ELISA indices for Dsg1 (A), Dsg3 (B), and BP180 (C) are shown for ELISA indices up to 1000 U/mL. CLEIA: chemiluminescent enzyme immunoassay, ELISA: enzyme-linked immunosorbent assay.

ELISA; one case in remission yielded a positive ELISA (23.0) and a negative CLEIA result (7.6 U/mL), and one case with active disease yielded a positive ELISA (55.0) and a negative CLEIA result (1.9 U/mL).

Table 2
 The correlation of indexes of ELISA and CLEIA.

	Dsg1	Dsg3	BP180
CLEIA/ELISA average	1.10	1.67	3.75
CLEIA > ELISA	48%	65%	86%
CLEIA/ELISA			
0.5–2.0	60%	56%	34%
0.2–5.0	79%	95%	76%
0.25–4.0	79%	93%	62%
0.125–8.0	87%	96%	94%
0.1–10.0	91%	98%	95%

CLEIA: chemiluminescent enzyme immunoassay, ELISA: enzyme-linked immunosorbent assay.

4. Discussion

We explored the ability of the CLEIA (a newly developed method) to detect autoantibodies in patients with PV, PF, and BP. We found that the CLEIA titers fluctuated with the clinical severity scores (PDAI and BPDAl) and with the ELISA indices over the course of the disease, suggesting that the CLEIA can be used to monitor disease activity similar to the ELISA. Our use of the PDAI and BPDAl as objective indicators of clinical manifestations in patients with autoimmune blistering diseases was important in terms of study reliability. This is the first study to feature long-term observations of the relationship between clinical scores and autoantibody titers. A longer-term study involving a larger cohort of patients with pemphigus and pemphigoid is required to confirm our outcomes. It is also necessary to consider the few cases in whom the CLEIA did not reflect clinical severity.

Table 3
 Concordance rate between CLEIA and ELISA

A. Dsg1				
		ELISA		
		positive	negative	
CLEIA	positive	73	0	73
	negative	2	30	32
		75	30	105
Concordance rate (%)				98
B. Dsg3				
		ELISA		
		positive	negative	
CLEIA	positive	59	0	59
	negative	1	45	46
		60	45	105
Concordance rate (%)				99
C. BP180				
		ELISA		
		positive	negative	
CLEIA	positive	47	0	47
	negative	2	3	5
		49	3	52
Concordance rate (%)				96

PV: pemphigus vulgaris, PF: Pemphigus foliaceus, BP: Bullous pemphigoid. CLEIA: chemiluminescent enzyme immunoassay, ELISA: enzyme-linked immunosorbent assay.

Another implication from our results is that the CLEIA and ELISA indices can differ substantially in the same sample, even though the same recombinant proteins were used as antigens. Although the CLEIA and ELISA indices for Dsg1, Dsg3, and BP180 correlated with each other in general (r^2 values of 0.80–0.85), we could not find a formula to convert ELISA indices into CLEIA titer values, or vice versa. This is important for clinicians managing patients with pemphigus and pemphigoid, because the CLEIA will replace the ELISA in most cases, as it is a new and more efficient autoantibody detection system. Our results suggested that the CLEIA/ELISA ratios for Dsg3 and BP180 tended to be higher than that for Dsg1. However, some patients had lower CLEIA than ELISA values for each target antigen, and it was impossible to predict the CLEIA titer from the ELISA index. It remains unclear why no correlation was seen between the CLEIA and ELISA indices in some cases, despite the same recombinant proteins used in both detection systems. Possible causes include differences in the dilution linearity, the conversion formulae used, the detection systems, and solid phase viscosities, as follows. Antibodies detected in each assay could be affected by different serum dilution that tended to be higher in ELISA. CLEIA has a wider dynamic range than ELISA and each assay uses different conversion formulae. While antigens are physically bound to the plate in ELISA, those are connected to the magnetic beads with covalent bond in CLEIA. Difference in binding to the solid phase between CLEIA and ELISA could cause antibodies' binding efficacy to antigens because of their steric position.

Although the CLEIA and ELISA values can differ substantially, the agreement in the positivity/negativity for an antigen between the CLEIA and ELISA results was high (94–99%). Three of 105 Dsg samples and 6 of 103 BP180 samples from patients in remission yielded negative CLEIA but positive ELISA results. In two BP cases with fresh blisters and in one PV case exhibiting exacerbation, ELISA (55.0/40.6) afforded more accurate information than did the CLEIA (1.9/7.3 U/mL). Except for these cases, the positive and negative results were in agreement between the CLEIA and ELISA. In practice, these few exceptions will not be of concern to clinicians, because all seven samples were obtained from patients lacking clinical activity, i.e., in the transient phase from a positive to negative result in terms of autoantibody detection.

Compared with ELISA, the CLEIA has the advantages of accuracy, stability, and efficiency, as it is fully automated. The CLEIA rapidly and reliably detected antibodies against cyclic citrullinated peptide in patients with rheumatoid arthritis [19], and CLEIA will become the standard method for quantifying autoantibodies in patients with autoimmune bullous diseases. We verified the utility and reliability of the CLEIA and showed that CLEIA values fluctuated with disease activity during the clinical courses of PF, PV, and BP. Although the CLEIA and ELISA values may differ substantially within an individual sample, the agreement in positive/negative results between the two methods is acceptably high.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jdermsci.2016.12.007>.

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