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FAST-TRACK PAPER

Usefulness of enzyme-linked immunosorbent assay using recombinant desmogleins 1 and 3 for serodiagnosis of pemphigus

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Summary Pemphigus is an autoimmune blistering disease with two major subtypes, pemphigus vulgaris (PV) and pemphigus foliaceus (PF). Patients with pemphigus have circulating antidesmoglein (Dsg)1 and/ or anti-Dsg3 IgG autoantibodies. We have previously developed enzyme-linked immunosorbent assays (ELISAs) using recombinant Dsg1 and Dsg3 expressed by baculovirus as a diagnostic tool for pemphigus. The purpose of this study was to evaluate the practical application of these ELISAs for clinical use with a large number of serum samples. We used 81 PV sera, 48 PF sera, 114 bullous pemphigoid (BP) sera, 124 collagen disease sera, nine sera of other non-pemphigus bullous diseases and 179 normal control sera. A cut-off value was determined by receiver-operating-characteristic plots. Forty-seven of 48 PF sera (97·9%) were positive in the Dsg1 ELISA and 79 of 81 PV sera (97·5%) were positive in the Dsg3 ELISA, while only two (1·1%) and four (2·2%) of 179 normal sera were positive in Dsg1 and Dsg3 ELISAs, respectively. However, some disease control sera of BP and collagen diseases exceeded the cut-off value. Introduction of a grey zone helped to decrease the number of these false-positive sera. Furthermore, in three patients studied, the respective Dsg1 and Dsg3 ELISA scores showed parallel fluctuation with the disease activity along the time course. We conclude that Dsg1 and Dsg3 ELISAs provide a simple, sensitive and highly specific assay for the diagnosis of patients with PV and PF and that these ELISAs may be a valuable tool to monitor the disease activity. We also propose diagnostic criteria for pemphigus based on ELISA reactivity: if a serum is positive against Dsg3 it indicates a diagnosis of PV, regardless of reactivity against Dsg1; if a serum is negative for Dsg3 and positive for Dsg1, it indicates a diagnosis of PF.

Key words: enzyme-linked immunosorbent assay, desmoglein 1, desmoglein 3, pemphigus, diagnosis.

Pemphigus has two major subtypes, pemphigus vulgaris (PV) and pemphigus foliaceus (PF). The hallmark of pemphigus is the finding of IgG autoantibodies against keratinocyte cell surfaces *in vivo* and *in vitro*. Demonstration of IgG autoantibodies against keratinocyte cell surfaces is essential to make a diagnosis of pemphigus. The method of detecting the autoantibodies largely relies on immunofluorescence testing utilizing both direct immunofluorescence (DIF) and indirect immunofluorescence (IIF). The cell staining pattern using DIF or IIF is virtually identical, making it difficult to distinguish between PV and PF.

The autoantigens for PV and PF have been identified as desmoglein (Dsg)3 and Dsg1, respectively. These are desmosomal cadherins and are considered to play an important role in cell–cell adhesion in the stratified squamous epithelia.[1–4] Availability of cDNA clones for the pemphigus antigens has allowed us to produce recombinant proteins which represent epitopes of the native antigens, including conformational ones.[5,6] Using these recombinant antigens (rDsg1 and rDsg3), we have developed a sensitive and highly specific

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enzyme-linked immunosorbent assay (ELISA) system for detection of autoantibodies against Dsg1 and Dsg3.[7]

The purpose of this study was to evaluate the practical application of these ELISA tests for the serological diagnosis for PV and PF with a large number of serum samples collected from four different dermatological institutions in Japan. We also simplified the calculation of ELISA scores. The demonstration of reactivity against either Dsg3 or Dsg1 indicates a diagnosis of pemphigus. Moreover, combined ELISA results against Dsg3 and Dsg1 allowed us to differentiate PV and PF. We propose diagnostic criteria based on ELISA reactivity, which should be very useful in daily dermatological practice.

Materials and methods

Human sera

All sera tested in this study were collected from four different dermatological institutions: Kurume University, Ehime University, Gifu University and Keio University. Sera were obtained from 81 patients with PV and 48 with PF. All the patients studied had typical clinical and histological features of each disease. These sera were obtained when the disease was active. One serum from a patient with PV and one from a patient with PF, which were available in large amounts, were used as a positive reference for the Dsg3 and Dsg1 ELISAs. Serial serum samples from two patients with PV and one patient with PF were obtained and used for correlation with disease activity. Disease activity was arbitrarily assessed on a scale of 0–4.

Control sera for bullous skin diseases were obtained from 114 patients with bullous pemphigoid (BP), whose diagnosis was confirmed by clinical criteria, routine histology and immunopathology. Other control sera for bullous skin diseases ($n = 9$) were obtained: linear IgA bullous dermatosis ($n = 3$), epidermolysis bullosa acquisita ($n = 2$), drug-induced bullous eruption ($n = 3$) and herpes gestationis ($n = 1$). Sera obtained from 124 patients with various collagen diseases, comprising 77 patients with systemic lupus erythematosus, 12 with Sjögren's syndrome, 14 with rheumatoid arthritis, 10 with dermatomyositis and 11 with mixed connective tissue diseases, were used as disease control sera. Sera obtained from 179 normal individuals were also used.

Desmoglein 3 and desmoglein 1 enzyme-linked immunosorbent assays

ELISA using rDsg1 and rDsg3 was performed as previously described with slight modification.[7] Briefly, secreted forms of rDsg1 and rDsg3, which include the entire extracellular domains of Dsg1 and Dsg3 with a His tag at the carboxyl-terminal end, were produced by baculovirus expression. High Five cells (Invitrogen, San Diego, CA, U.S.A.) cultured in serum-free EX Cell 405 medium (JRH Bioscience, Lenexa, KS, U.S.A.) were infected with the recombinant viruses and incubated for 3 days. Recombinant baculoproteins were produced in the culture supernatant. Supernatants were stored at − 70 °C after removal of cell debris by centrifugation. The baculoproteins were purified on TALON (Clontech, Palo Alto, CA, U.S.A.) affinity metal resin according to the manufacturer's recommendations. Microtitre 96-well plates were coated with 100 μL of 5 μg/mL purified rDsg1 or rDsg3 at 4 °C overnight. The plates were kept at 4 °C until use after dry up.

All sera were diluted 200-fold and incubated for 1 h at room temperature on the rDsg-coated 96-well ELISA plates. After washing three times with phosphate-buffered saline with 0·13% Tween 20, pH 7·3, the plates were then incubated with horseradish peroxidase (HRP)-conjugated mouse monoclonal antihuman IgG antibody (MBL, Nagoya, Japan) for 1 h at room temperature. After washing, the colour development was achieved by mixing solution A (10 mmol/L trisodium citrate dihydrate, 1 mmol/L tetramethylbenzidine [TMB], 20% dimethylformamide and 1·25% polyethylene glycol) and solution B (10 mmol/L citric acid monohydrate and 4 mmol/L hydrogen peroxide). TMB is oxidized in the presence of HRP and results in a detectable colour change which is related to the amount of HRP and thus indirectly to the quantity of anti-Dsg antibodies. The mixed solution was incubated for 30 min at room temperature and the colour development was stopped by adding 1 mol/L H_2SO_4. The absorbance was measured at 450 nm by microtitre plate reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

The index value was defined by the following formula: index = (optical density [OD] of tested serum − OD of negative control)/(OD of positive control − OD of negative control) × 100. A positive and negative control were included in every plate and these controls were the same on every plate. Positive controls for Dsg1 and Dsg3 ELISAs were a diluted standard PF and PV serum, respectively. The negative control was a diluted standard serum obtained from a normal individual. The negative control revealed the non-specific, background noise of the system, which was subtracted from all values on the plate. Adjusting the values relative to the positive control allows comparison of results from different plates,

even when run on a different day under different ambient conditions and even in different laboratories. The index values can all be calculated from the raw data with a calculator, without the need for any expensive software, unlike other quantification techniques. The whole test took less than 3 h to obtain the results.

Statistical analysis

A receiver-operating-characteristic (ROC) analysis was performed to determine a cut-off value for Dsg1 and Dsg3 ELISAs. The sensitivity and specificity were plotted when different scores (0–240) were used for cut-off values and the one which gave the highest score for the sum of sensitivity (%) and specificity (%) was determined to be the cut-off value to be used. For this analysis, data obtained with PF sera and normal control sera were used for Dsg1 ELISA and data obtained with PV sera and normal control sera were used for Dsg3 ELISA.

Results

Determination of cut-off values and grey zone

We examined 48 PF sera and 179 normal sera with Dsg1 ELISA, and 81 PV sera and 179 normal sera with Dsg3 ELISA. A ROC analysis was performed to determine a cut-off value which differentiates positive and negative reactions (Fig. 1). The one which gave the highest sum of sensitivity (%) and specificity (%) was defined as the cut-off value to be used. For Dsg1 and Dsg3 ELISAs, this was determined to be 11·0 and 10·0, respectively. With this cut-off value, the sensitivity and specificity of Dsg1 ELISA were 97·9% (47/48) and 98·9% (177/179), respectively. Those for Dsg3 were 97·5% (79/81) and 97·8% (175/179). The grey zone was defined as being between the cut-off value and 20·0 to differentiate false-positive and true-positive sera as much as possible, as discussed below.

When serum samples of four different concentrations were assayed repeatedly eight times, the coefficient of variation was < 15% (data not shown). When all reagents were stored at 4 °C, ELISA reactivity was stable for 6 months after production (data not shown).

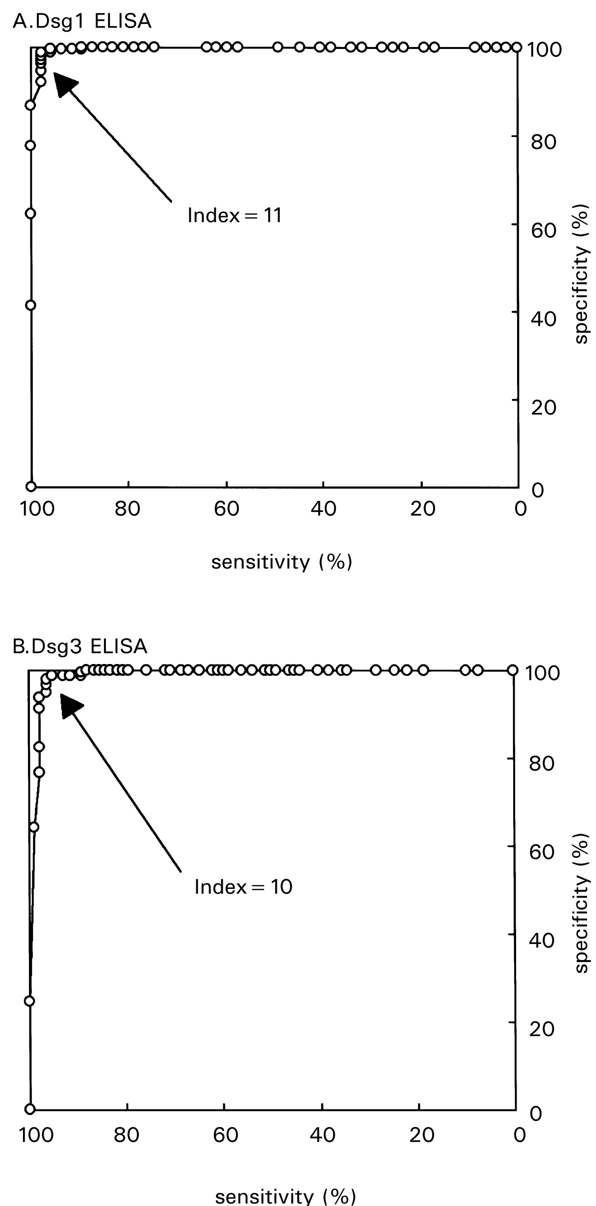

Figure 1. Receiver-operating-characteristic curves to determine a cut-off value for desmoglein (Dsg)1 (A) and Dsg3 (B) enzyme-linked immunosorbent assays (ELISAs).

Analysis of sera obtained from patients and controls with desmoglein 1 and desmoglein 3 enzyme-linked immunosorbent assays

For Dsg1 ELISA, 47 of 48 sera from PF patients (97·9%) and 56 of 81 sera from PV patients (69·1%) exceeded the cut-off value, while nine of 114 sera from BP patients (7·9%), five of 124 sera from patients with various collagen diseases (4·0%), one of nine sera from patients with other bullous diseases (11·1%) and two of 179 normal control sera (1·1%) exceeded the cut-off value (Fig. 2, Table 1). For Dsg3 ELISA, 79 of 81 sera from PV patients (97·5%) showed positive scores above the cut-off value, compared with 12 of 48 sera from PF patients (25·0%), 22 of 114 sera from BP patients (19·3%), eight of 124 sera from patients with collagen

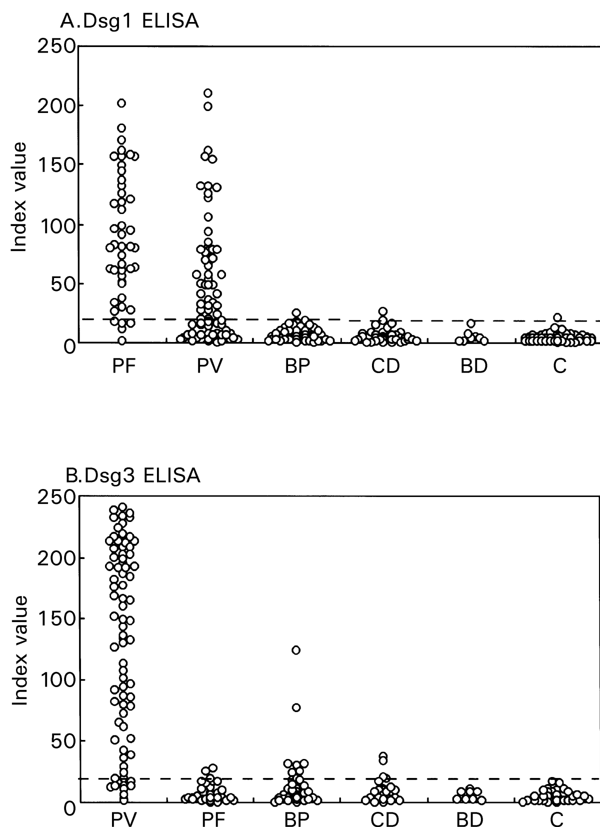

Figure 2. Antidesmoglein (Dsg)1 (A) and anti-Dsg3 (B) IgG titres obtained by enzyme-linked immunosorbent assays (ELISAs) using recombinant Dsgs. PV, pemphigus vulgaris; PF, pemphigus foliaceus; BP, bullous pemphigoid; CD, collagen diseases; BD, other bullous diseases; C, normal control sera. Dashed lines indicate the index value 20·0 for the upper limit of the grey zone.

diseases (6·5%), one of nine sera from patients with other bullous diseases (11·1%) and four of 179 normal control sera (2·2%).

Most of the positive disease control sera of BP, collagen diseases and other bullous diseases barely exceeded the cut-off values on Dsg1 or Dsg3 ELISA. None of these sera, including two BP sera with high index values, showed any apparent cell surface staining on IIF with normal human skin. Therefore, we concluded that those sera showed false-positive reactivity and decided to introduce a grey zone.

When the index value 20·0 was used to differentiate positive and negative reactions, on Dsg1 ELISA, 43 of 48 sera from PF patients (89·6%) and 45 of 81 sera from PV patients (55·6%) were positive, while one of 114 sera from BP patients (0·9%), one of 124 sera from patients with various collagen diseases (0·8%), none of nine sera from patients with other bullous diseases (0%) and one of 179 normal control sera (0·6%) exceeded the index value 20·0. For Dsg3 ELISA, 69 of 81 sera from PV patients (85·2%) showed positive scores above the index value 20·0, compared with two of 48 sera from PF patients (4·2%), eight of 114 sera from BP patients (7·0%), three of 124 sera from patients with collagen diseases (2·2%), none of nine sera from patients with other bullous diseases (0%) and none of 179 normal control sera (0%).

Diagnostic criteria for pemphigus foliaceus and pemphigus vulgaris based on results of enzyme-linked immunosorbent assay

One of the disadvantages of conventional immunofluorescence using normal human skin or monkey oesophagus as substrate is the difficulty of differentiating PF and PV from the staining pattern. However, because this set of ELISAs is able to measure antibodies against specific target molecules for PF and PV, most pemphigus sera can be diagnosed as PF or PV by the following criteria (Table 2): if a serum tested is positive against Dsg3 it indicates a diagnosis of PV, regardless of reactivity against Dsg1; if a serum tested is negative for Dsg3 and positive for Dsg1, it indicates a diagnosis of PF.

Table 1. Positive ratio of examined sera by desmoglein (Dsg) 3 and Dsg1 enzyme-linked immunosorbent assays (ELISAs)

		ELISA		ELISA	
Disease	*n*	Dsg1 Cut-off at 11·0	Dsg3 Cut-off at 10·0	Dsg1 Cut-off at 20·0	Dsg3 Cut-off at 20·0
Pemphigus vulgaris	81	56 (69·1%)	79 (97·5%)	45 (55·6%)	69 (85·2%)
Pemphigus foliaceus	48	47 (97·9%)	12 (25·0%)	43 (89·6%)	2 (4·2%)
Bullous pemphigoid	114	9 (7·9%)	22 (19·3%)	1 (0·9%)	8 (7·0%)
Collagen diseases	124	5 (4·0%)	8 (6·5%)	1 (0·8%)	3 (2·2%)
Other bullous diseases	9	1 (11·1%)	1 (11·1%)	0 (0·0%)	0 (0·0%)
Normal	179	2 (1·1%)	4 (2·2%)	1 (0·6%)	0 (0·0%)

Table 2. Diagnostic criteria for pemphigus based on the enzyme-linked immunosorbent assay results

	Desmoglein 1	Desmoglein 3
Pemphigus vulgaris	+ or −	+
Pemphigus foliaceus	+	−

We applied these criteria to the cases of pemphigus examined in this study, considering that an index value greater than 20·0 was positive for this analysis. Out of 48 PF sera, 43 were positive against Dsg1, but negative against Dsg3, which serologically indicates PF. Of 81 PV sera, 69 were positive against Dsg3, which serologically indicates PV. Therefore, serodiagnosis by ELISA matched the clinical diagnosis in 86·8% of PV and PF cases tested in this study. Most of the sera which did not match the clinical diagnosis had negative reactivity against the respective primary antigen, but not cross-reactivity to the wrong antigen.

Enzyme-linked immunosorbent assay scores to monitor the disease activity

We also examined the correlation between disease activity of pemphigus over time and ELISA scores. We tested two patients with PV and one patient with PF (Fig. 3).

The first PV patient was a 53-year-old Japanese woman with predominant oral erosions and minimal skin involvement. She was started on 60 mg/day of prednisolone with rapid improvement of the oral lesions. The prednisolone was tapered over 40 days to 20 mg/day and maintained at 20 mg/day for the following 3 months. Then, a flare of oral lesions was noted and cyclosporin was added at 5 mg/kg per day. Dsg3 ELISA scores decreased in parallel with the disease activity and increased when the patient had the flare. In contrast, Dsg1 ELISA scores continued to be negative until 3 June 1997, and turned positive with the flare (Fig. 3A).

The second PV patient was a 69-year-old Japanese man with extensive oral erosions and scattered skin blisters and erosions. Treatment was started with plasmapheresis (four times a week for two rounds) in addition to 6 mg/day of oral betamethasone. The oral and cutaneous lesions gradually cleared and the betamethasone was tapered over 8 months to 2 mg/day without any apparent flares. In this case, Dsg3 ELISA scores dropped dramatically with the initiation of treatment. We assumed that this drop was due to plasmapheresis. However, ELISA scores usually go up within a couple of days after plasmapheresis (M.Amagai, unpublished data). Unfortunately, this rebound phenomenon could not be measured in this case because serum samples were not available for that period. The Dsg3 ELISA score on 12 December 1996 was higher than that just after the plasmapheresis, and then gradually decreased as the disease subsided. Dsg1 ELISA scores remained negative throughout.

The PF patient was a 50-year-old Japanese woman with scaly crusted erythema and erosions on her face and trunk. Oral prednisolone at 30 mg/day was initiated without apparent improvement. Pulse therapy with intravenous methylprednisolone, 1 g/day for 3 days, was added with rapid clear-up of skin lesions. The prednisolone was tapered over 5 months to 17·5 mg/day without apparent flares, but she then had a relapse with extensive skin lesions. For this patient, the Dsg1 ELISA score was initially high, then gradually decreased as the disease subsided and went up again at the time of relapse. Dsg3 ELISA scores were negative throughout.

Discussion

We evaluated the Dsg1 and Dsg3 ELISA tests with a large number of serum samples obtained from patients with pemphigus, patients with other diseases and normal individuals. We simplified the calculation of ELISA scores,[7] and used index values which are a ratio of reactivity to a standard positive serum. We first used PF, PV and normal sera to analyse the sensitivity and specificity using ROC curves. The cut-off values for achieving highest sensitivity and specificity were determined to be 11·0 for Dsg1 ELISA and 10·0 for Dsg3 ELISA. However, with these cut-off values, a small number of sera obtained from other bullous diseases or collagen diseases fell into the positive zone. To decrease the ratio for those false-positive sera, we set up a grey zone, which is above the cut-off value and below 20·0. With this setting, the number of positive sera in disease control groups dramatically dropped, although this setting decreased the positive ratio of pemphigus sera. Regarding the assessment of the reactivity, we suggest the following categorization: for Dsg1 ELISA, an index value which is 11·0 or lower is negative, an index value which is higher than 11·0 but 20·0 or lower is ±, and an index value higher than 20·0 is positive. For Dsg3 ELISA, an index value which is 10·0 or lower is negative, an index value which is

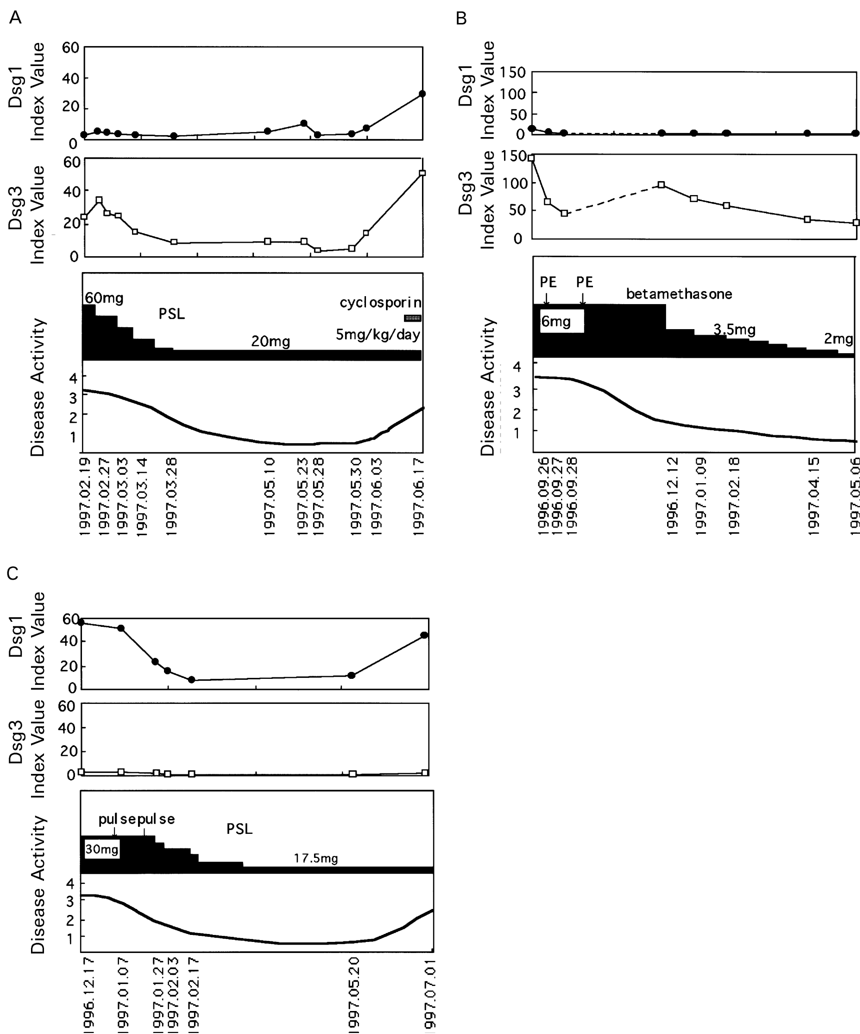

Figure 3. Desmoglein (Dsg) 1 and Dsg3 enzyme-linked immunosorbent assay scores fluctuate in parallel with the disease activity over a time course. Two pemphigus vulgaris patients (A, B) and one pemphigus foliaceus patient (C) were studied. PE, plasmapheresis; pulse, methylprednisolone pulse therapy; PSL, prednisolone.

higher than 10·0 but 20·0 or lower is ±, and an index value higher than 20·0 is positive.

In this study, we have proposed diagnostic criteria for PV and PF using ELISA results (Table 2). The majority of sera follow these criteria. However, sera obtained from patients in remission may not necessarily do so. For example, we observed that some sera obtained from patients with PV in remission showed only reactivity against Dsg1 but not against Dsg3 (unpublished observation). We speculate that these patients used to have both anti-Dsg3 and anti-Dsg1 autoantibodies when the disease was active, but as the disease subsided anti-Dsg3 antibodies became negative while anti-Dsg1 antibodies stayed positive. Therefore, the diagnostic criteria apply only to patients who have active disease.

We used these ELISAs to quantify individual levels of antibodies against Dsg1 and Dsg3 and examined their correlation with the progression of disease activity. In the three patients studied, the respective Dsg1 and Dsg3 ELISA scores showed parallel fluctuation with the disease activity along the time course. These observations suggest that these ELISAs will be a valuable tool to monitor the disease activity. ELISA scores may be useful to plan tapering schedules of corticosteroids and to predict flares or relapses by detecting increases in antibodies before clinical evidence of disease flares are noticed. Further prospective studies will be needed to confirm this.

In summary, the Dsg1 and Dsg3 ELISAs using recombinant pemphigus antigens, when used together, provide a sensitive, specific and quantitative diagnostic tool for the detection of anti-Dsg1 and anti-Dsg3 autoantibodies. This new diagnostic tool will be useful in dermatology clinics to make a proper diagnosis and evaluate patients with pemphigus as well as in

laboratories to understand the fundamental pathophysiological mechanisms of pemphigus.

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References

1 Amagai M, Klaus-Kovtun V, Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell* 1991; **67:** 869–77.
2 Stanley JR. Cell adhesion molecules as targets of autoantibodies in pemphigus and pemphigoid, bullous diseases due to defective epidermal cell adhesion. *Adv Immunol* 1993; **53:** 291–325.
3 Amagai M. Pemphigus: autoimmunity to epidermal cell adhesion molecules. *Adv Dermatol* 1996; **11:** 319–52.
4 Koch PJ, Mahoney MG, Ishikawa H *et al*. Targeted disruption of the pemphigus vulgaris antigen (desmoglein 3) gene in mice causes loss of keratinocyte cell adhesion with a phenotype similar to pemphigus vulgaris. *J Cell Biol* 1997; **137:** 1091–102.
5 Amagai M, Hashimoto T, Shimizu N *et al*. Absorption of pathogenic autoantibodies by the extracellular domain of pemphigus vulgaris antigen (Dsg3) produced by baculovirus. *J Clin Invest* 1994; **94:** 59–67.
6 Amagai M, Hashimoto T, Green KJ *et al*. Antigen-specific immunoadsorption of pathogenic autoantibodies in pemphigus foliaceus. *J Invest Dermatol* 1995; **104:** 895–901.
7 Ishii K, Amagai M, Hall RP *et al*. Characterization of autoantibodies in pemphigus using antigen-specific ELISAs with baculovirus expressed recombinant desmogleins.*J Immunol* 1997; **159:** 2010–17.

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