Detection of antibodies against the non-calcium-dependent epitopes of desmoglein 3 in pemphigus vulgaris and their pathogenic significance

Running head: Detection of pathogenic pemphigus antibodies

Abstract word: 250 words

Manuscript word: 2958 words

Table count: 2

Figure count: 8


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Funding sources: This work was supported by a grant from the Ministry of Health, Labour and Welfare (Research for Intractable Diseases), and a Ministry of Education, Culture, Sports, Science and Technology.

Conflicts of interest: None declared.

Abbreviations: Dsg; desmoglein, PV; pemphigus vulgaris, Ca$^{2+}$; calcium, ELISA; enzyme-linked immunosorbent assay, EDTA; ethylenediaminetetraacetic acid, mAbs; monoclonal antibodies, EC; extracellular domains, PBS; phosphate buffered saline, PBS-Ca; 1 mM CaCl$_2$ in PBS

What’s already known about this topic?
Pathogenic anti-desmoglein (Dsg) 3 monoclonal antibodies (mAbs) recognize the calcium (Ca\(^{2+}\))-dependent conformational epitopes.

Pemphigus vulgaris (PV) sera contain antibodies against a variety of different epitopes of Dsg3.

The combination of mAbs against the different epitopes induced stronger pathogenicity than a single mAb.

Anti-Dsg3 serum antibody titers are usually correlated with the disease activity of PV, but some patients have nonpathogenic anti-Dsg3 serum antibodies.

What does this study add?

We have developed ethylenediaminetetraacetic acid (EDTA) treated enzyme-linked immunosorbent assay (ELISA), which can distinguish anti-Dsg3 antibodies against Ca\(^{2+}\)-dependent from non-Ca\(^{2+}\)-dependent epitopes easily.

EDTA-treated ELISA revealed that the proportion of anti-Dsg3 serum antibodies against the Ca\(^{2+}\)-dependent epitopes decreased after entering the inactive phase in PV patients.
EDTA-treated ELISA can detect nonpathogenic anti-Dsg3 serum antibodies against the non-Ca^{2+}-dependent epitopes, and also be useful for monitoring the disease activity in PV patients.

(69 words)
Abstract

Background Anti-desmoglein (Dsg) 3 serum antibody titers are usually correlated with the disease activity of pemphigus vulgaris (PV), but some patients retain high titers even in remission.

Objectives The aim of our study was to determine whether anti-Dsg3 antibodies in PV sera recognized calcium (Ca^{2+})-dependent or non-Ca^{2+}-dependent epitopes, and evaluate their pathogenicity.

Methods Dsg3 baculoprotein-coated enzyme-linked immunosorbent assay (ELISA) plates were treated with 0.5 mM ethylenediaminetetraacetic acid (EDTA). The binding ability of anti-Dsg3 monoclonal antibodies (mAbs) was analyzed. Eight of the 83 PV patients screened had elevated Dsg3 ELISA index values greater than 100 index values in remission. The binding ability of those PV sera was analyzed. We evaluated the pathogenicity of anti-Dsg3 serum antibodies against the non-Ca^{2+}-dependent epitopes using dissociation assay.

Results The reactivity of pathogenic anti-Dsg3 mAbs against the Ca^{2+}-dependent epitopes diminished markedly in EDTA-treated ELISA, whereas no such reduction was observed in mAbs against the non-Ca^{2+}-dependent epitopes. All patients' sera contained antibodies
against both Ca$^{2+}$-dependent and non-Ca$^{2+}$-dependent epitopes. In 6 out of the 8 patients, the ratio of antibodies against Ca$^{2+}$-dependent to non-Ca$^{2+}$-dependent epitopes decreased in remission. EDTA-treated Dsg3 baculoproteins adsorbed anti-Dsg3 serum antibodies against the non-Ca$^{2+}$-dependent epitopes, but the remnant PV antibodies retained the ability to induce acantholysis in dissociation assay.

Conclusions We have established an assay to indirectly measure the titers of anti-Dsg3 serum antibodies against the Ca$^{2+}$-dependent epitopes, which are the differences between EDTA-untreated and EDTA-treated ELISA index values, as a routine laboratory test to reflect the pathogenic anti-Dsg3 serum antibody titers more accurately.
**Introduction**

PV is an autoimmune blistering disease, characterized by the loss of cell-cell adhesion between epidermal keratinocytes and the presence of autoantibodies against Dsg3. The binding of autoantibodies to Dsg3 causes endocytosis of Dsg3 from the cell surface, and results in the formation of Dsg3-depleted desmosomes which is weak adhesive desmosome, and can lead to desmosomal dissociation. **However, the mechanisms of desmosomal dissociation are not fully understood and multiple pathways may be involved in acantholysis in a coordinated manner.**

Dsgs, desmosomal members of the cadherin superfamily, contain four cadherin repeats of approximately 110 amino acids (extracellular domains (EC) 1-4) and an extracellular anchor (EC5) in the amino-terminal ectodomain. The adhesive properties of cadherin-family proteins mainly involve the EC1 and EC2, and the junctions between these domains are characterized by Ca\(^{2+}\) binding sites. Ca\(^{2+}\) is essential for the correct functioning of cadherins. A previous study showed that pathogenic AK23 and AK19 mAbs which bind to the EC1-2 of Dsg3 in a Ca\(^{2+}\)-dependent manner were capable of inducing acantholysis, while other mAbs AK18, AK15, and AK20 which bind to the EC2-5 Ca\(^{2+}\)-independently were not. Interestingly, anti-Dsg3 antibodies which bind to
the non-Ca\textsuperscript{2+}-dependent epitopes did not induce acantholysis alone, but enhanced acantholysis synergistically in the presence of anti-Dsg3 antibodies against the Ca\textsuperscript{2+}-dependent epitopes \textsuperscript{13}. Therefore, it is hard to avoid the impression that the epitopes of antibodies should be a key factor in their pathogenicity as well as their titers.

In PV patients, the titers of anti-Dsg serum antibodies, as measured by conventional ELISA, generally correlate with the disease activity, and are proportional to the severity of the disease, especially when monitored in individual patients \textsuperscript{14-15}. In some cases, however, anti-Dsg3 serum antibody titers remain high even though the disease is clinically inactive \textsuperscript{16-17}. Conventional ELISA is a serological laboratory test for determining the binding capacity of autoantibodies against a whole Dsg3 molecule containing the EC1-5, and cannot distinguish the epitopes. In order to address the discrepancy between anti-Dsg3 serum antibody titers and the clinical course, we analyzed the epitope types recognized by anti-Dsg3 antibodies in PV sera. We distinguished the titers of anti-Dsg3 antibodies against Ca\textsuperscript{2+}-dependent epitopes from non-Ca\textsuperscript{2+}-dependent epitopes using commercially available Dsg3 ELISA plates with EDTA-treatment. Because Ca\textsuperscript{2+} maintains the conformational structure of Dsg3, the ELISA plate was treated with EDTA so that the conformational structure of Dsg3 coated on the ELISA plate could be modified. We
examined whether this assay was more sensitive than conventional ELISA at detecting pathogenic antibodies in both in vitro and clinical situations.

**Materials and methods**

**Anti-Dsg3 mAbs**

Anti-Dsg3 mAbs (AK15, 18, 19, 20, 23) were produced by hybridoma cells from splenocytes isolated from PV model mice, as previously described 12.

**PV patients’ sera**

We retrospectively reviewed 83 PV patients who were treated between January 2003 and December 2009. Serum samples stocked during the clinical courses were used. Of 83 PV patients screened, 8 patients had elevated Dsg3 ELISA index values greater than 100 index values in the inactive phase, which was defined as the absence of new and/or established lesions on the skin and mucosa while treated with 10 mg/day of prednisolone or less (or the equivalent) for at least 2 months, according to the consensus statement 18.

**EDTA-treated ELISA and conformational ELISA index value**
Immunoreactivity was evaluated using Dsg ELISA according to the kit manufacturer’s instructions (MBL, Nagoya, Japan). In order to modify the Ca\(^{2+}\)-dependent epitopes of Dsg3 molecules coated on the ELISA plate, the plates were treated with 0.5 mM EDTA for 30 minutes at room temperature as previously reported\(^{19}\). After washing 4 times with Dsg ELISA wash buffer, the conventional assay protocol was carried out. The modified structure of Dsg3 molecules cannot be restored after EDTA-treatment because kit reagents are free from Ca\(^{2+}\) throughout the ELISA process. Both EDTA-untreated and EDTA-treated ELISA were performed with same samples at once. The differences between EDTA-untreated and EDTA-treated ELISA index values were calculated as conformational ELISA index values. Conformational ELISA index values did not reflect antibodies against the non-Ca\(^{2+}\)-dependent epitopes, but against the Ca\(^{2+}\)-dependent epitopes.

**IgG purification**

IgG fractions were isolated using HiTrap Protein G columns (GE Healthcare, Tokyo, Japan). ELISA index values of purified IgG containing 30 µg protein were determined.

**Dissociation assay**
The adhesive strength of DJM-1 cell-cell contacts was assayed based on an adapted method that modified previously described methods \(^{13,20-21}\). DJM-1 cells, a cell line isolated from human skin squamous cell carcinoma \(^{22}\), were seeded in 12 well dishes and cultured until they reached confluence \(^{13}\). The cells were then stimulated with 300 μg of purified PV-IgG and incubated at 37°C for 8 hours or 24 hours. For the dose-response experiments, each well was filled with 1 ml of medium containing PV-IgG ranging from 0.032 to 1000 μg/ml. After washing with PBS twice, cells were incubated with dispase for 30 minutes to detach the cell sheet from the bottom of the dish, and mechanical stress was applied by pipetting the cells 5 times with a P1000 Pipetman. The resulting dissociated cells were fixed by adding formaldehyde to a final concentration of 3%, stained with crystal violet. Then, the dishes were centrifuged at 2000 g for 5 minutes and the number of cell fragmentations was counted. Each experiment was carried out in triplicate.

**Ca\(^{2+}\)-supplemented and Ca\(^{2+}\)-depleted Dsg3 baculoproteins**

Dsg3 recombinant proteins were produced as previously described \(^{12,23}\). To examine the effects of Ca\(^{2+}\)-depletion, we added 5 mM EDTA to supernatants containing Dsg3 baculoproteins. Samples were incubated for 1 hour at room temperature and then dialyzed
against phosphate buffered saline (PBS) without Ca\textsuperscript{2+}. To determine whether the effects associated with Ca\textsuperscript{2+}-depletion were reversible, samples were dialyzed against 1 mM CaCl\textsubscript{2} in PBS (PBS-Ca) after 1-hour incubation at room temperature with 5 mM EDTA.

**Adsorption of the PV serum to Dsg3 baculoproteins**

To examine the effects Ca\textsuperscript{2+}-supplemented and Ca\textsuperscript{2+}-depleted Dsg3 baculoproteins have on adsorption, PV-IgG containing 300 µg protein were incubated with each Dsg3 baculoprotein containing 18000 µg protein for 30 minutes at room temperature, then subjected to dissociation assay, as described above. Each experiment was carried out in duplicate.

**Results**

**Effects of EDTA-treatments to the Dsg3 molecules on the ELISA plate**

To characterize the conformational change of 0.5 mM EDTA-treated Dsg3 recombinant protein, we first examined the affinity of five AK mAbs (Figs 1-2). The reduction rate of AK23 and AK19 mAbs, which bind to the EC1-2 of Dsg3, were 65.5–84.4% and 72.3–79.1% respectively. AK18 and AK15 mAbs, which bind to the EC2-4, showed a 72.3–79.1% and
11.8–43.9% reduction respectively. The decrease observed with AK18 mAb was equal to AK23 and AK19 mAbs, whereas the decrease observed with AK15 mAb was not as high as AK23 and AK19 mAbs. It is noteworthy that AK20 mAb, which binds to the EC4-5, did not show any reduction at all. The reduction rate of all AK mAbs was almost the same when ELISA plates were pretreated with 0.5 mM EDTA for 60 minutes or 5 mM EDTA for 30 minutes (data not shown). These results suggest that EDTA-treatment changed the whole structure of EC1-2 recognized by AK23 and AK19 mAbs, and a part of EC2-4 recognized by AK18 and AK15 mAbs, but could not change the structure of EC4-5 recognized by AK20 mAb.

**Immunoreactivity of PV patients’ sera against the EDTA-treated recombinant Dsg3**

We next analyzed the composition of anti-Dsg3 antibodies detected in PV patients’ sera. We performed Dsg3 ELISA with and without EDTA-treatment using 8 PV patients’ sera (Table 1). Anti-Dsg3 serum antibody titers, indicated as conventional ELISA index values, decreased but remained high after entering the inactive phase in 6 patients (Fig. 3, Table 1, cases 1, 2, 3, 6, 7, and 8). On the other hand, anti-Dsg3 serum antibody titers increased in 2 patients (Fig. 3, Table 1, cases 4 and 5), even in the inactive phase. Anti-Dsg3 serum
antibody titers in these cases did not correlate with the disease activity. In cases 1, 2, 3, 6, 7, and 8, both conventional Dsg3 ELISA index values and conformational Dsg3 ELISA index values were decreased in the inactive phase (Fig. 3, Table 1). By contrast, in cases 4 and 5, conventional Dsg3 ELISA index values were increased, whereas conformational Dsg3 ELISA index values were decreased. The ratio of anti-Dsg3 serum antibodies recognizing Ca$^{2+}$-dependnet and non-Ca$^{2+}$-dependent epitopes was determined (Fig. 4). Anti-Dsg3 serum antibodies against the non-Ca$^{2+}$-dependent epitopes were detected at a higher rate in the inactive phase. The proportion of antibodies against the Ca$^{2+}$-dependent epitopes decreased in 6 out of the 8 patients in the inactive phase. By contrast, the proportion of antibodies against the Ca$^{2+}$-dependent epitopes increased in 2 patients, cases 7 and 8. All patients’ sera contained anti-Dsg3 serum antibodies against both Ca$^{2+}$-dependent and non-Ca$^{2+}$-dependent epitopes.

**Evaluation of pathogenic correlation of conformational ELISA index value as examined by dissociation assay.**

To evaluate the pathogenic correlation of conformational ELISA index value in clinical cases, the pathogenicities of purified IgG from PV patients’ sera with same conventional Dsg3 ELISA index values but different EDTA-treated Dsg3 ELISA index values were
compared by dissociation assay (Table 2a). A serum of case 1 was obtained in the active phase, while a serum of case 2 was obtained in the inactive phase. In case 1, anti-Dsg3 serum-IgG showed 33.6 index value by conventional ELISA and 8.9 index value by EDTA-treated ELISA. Calculated conformational Dsg3 ELISA index value was 24.7 index value. Therefore, total anti-Dsg3 antibodies contained approximately 73.5 % of IgG against the Ca\textsuperscript{2+}-dependent epitopes. By contrast, anti-Dsg3 serum-IgG of case 2 contained no IgG against the Ca\textsuperscript{2+}-dependent epitopes. DJM-1 cells, cultured as monolayer sheets, were incubated for 8 hours with each purified IgG containing 0.032, 0.16, 0.8, 4, 100, 500, and 1000 μg/ml. IgG of PV case 1 showed dose-dependent reactivity between 4 and 1000 μg/ml, whereas IgG of PV case 2 did not induce fragmentations of the cell sheet at all (Fig. 5). As for IgG of PV case 2, fragmentations of the cell sheet were not induced despite the incubation for 24 hours (data not shown). These results indicated that conformational ELISA index values reflected the pathogenicity of anti-Dsg3 serum-IgG.

We next examined the effects of anti-Dsg3 serum-IgG against the non-Ca\textsuperscript{2+}-dependent epitopes on disruption of intercellular adhesion. Purified IgG from 4 serum samples of 2 PV patients with same conformational Dsg3 ELISA index values but different EDTA-treated Dsg3 ELISA index values were examined by dissociation assay (Table 2b).
Cell sheets were incubated with each purified IgG for 8 hours (Fig. 6a) and 24 hours (Fig. 6b). The number of cell fragmentations incubated with IgG No. 1-4 did not increase after the incubation for 8 hours. However, the number of cell fragmentations incubated with IgG No. 1 and 2 increased after the incubation for 24 hours in similar extent. On the other hand, the number of cell fragmentations incubated with IgG No. 3 and 4 did not increase at all despite the incubation for 24 hours. These results indicated that anti-Dsg3 serum-IgG against the non-Ca²⁺-dependent epitopes did not enhance the pathogenicity in dissociation assay.

**Pathogenicity of autoantibodies against the non-Ca²⁺-dependent epitopes of Dsg3 as examined by dissociation assay**

To elucidate the pathogenicity of antibodies against the non-Ca²⁺-dependent epitopes of Dsg3, we performed dissociation assay using the PV-IgG adsorbed with Dsg3 baculoproteins with or without EDTA-treatment. We used anti-Dsg3 serum-IgG, which showed 278.6 index value by conventional ELISA and 178.9 index value by conformational ELISA. The alteration of ELISA index values of anti-Dsg3 serum-IgG after immunoabsorption with each Dsg3 baculoprotein was analyzed. Both untreated Dsg3...
baculoproteins and EDTA-treated Dsg3 baculoproteins dialyzed against PBS-Ca completely blocked the immunoreactivity with 30µg of anti-Dsg3 serum-IgG at the higher amounts than 1800 µg (Fig. 7). By contrast, 1800 µg of EDTA-treated Dsg3 baculoproteins was sufficient to achieve complete immunoadsorption of antibodies against the non-Ca²⁺-dependent epitopes of Dsg3, because the immunoreactivity reached nearly equal to conformational Dsg3 ELISA index value. These adsorptive effects were also observed as examined by indirect immunofluorescence (data not shown). Cultured keratinocytes were stimulated for 24 hours. Cell sheets, incubated with EDTA-treated Dsg3-adsorbed anti-Dsg3 serum-IgG, dissociated into numerous smaller fragments, with the number of fragments nearly equal to that of the positive control (Fig. 8). These results indicated that EDTA-treated Dsg3 baculoproteins did not block the pathogenicity of anti-Dsg3 serum-IgG, and antibodies against the non-Ca²⁺-dependent epitopes of Dsg3 did not have an intimate involvement in the fragmentation of dissociation assay. On the other hand, untreated Dsg3-adsorbed anti-Dsg3 serum-IgG did not cause fragmentations of the cell sheets. The same result was obtained after treatment with Dsg3 baculoproteins dialyzed against PBS-Ca.
Discussion

To characterize the Dsg3 epitopes involved in binding, we first examined five anti-Dsg3 mAbs (AK15, 18, 19, 20, 23). EDTA-treatment reduced the reactivity of all AK mAbs except for AK20 mAb. The reactivity of each AK mAb also varied with its concentration. It is possible that each AK mAb has a different binding affinity for the Ca$^{2+}$-dependent epitopes of Dsg3. Our finding that AK23 mAb associates with Dsg3 at low concentrations demonstrates that it has a high affinity for Dsg3. The epitopes of AK23 and AK19 mAbs are present in the EC1-2 of Dsg3 (Fig. 1), and AK23 and AK19 mAbs could not bind to the EDTA-treated Dsg3. These results indicate that the conformational structures of the EC1-2 of Dsg3 are Ca$^{2+}$-dependent. The epitopes of AK18 and AK15 mAbs are present in the EC2-4 (Fig. 1). The binding of AK18 and AK15 mAbs was also diminished for the EDTA-treated Dsg3. However, as the concentration of the antibodies increased, the binding of these AK mAbs to Dsg3 also increased, suggesting that Ca$^{2+}$-dependent conformational structures of the epitopes recognized by AK18 and AK15 mAbs were a little changed because AK18 and AK15 mAbs bound to the EDTA-treated ELISA plates with less affinity.

The pathogenesis of pemphigus is thought to be defined by various factors, such as antibody titer, the epitopes involved in binding, and the predominant IgG subclasses.
involved. In some PV patients, Dsg3 ELISA index values remain high even in remission
suggesting that positive ELISA index values reflect the nonpathogenic anti-Dsg3
serum antibodies. We examined sera obtained from 8 PV patients whose ELISA index
values were persistently high in remission. All patients’ sera contained antibodies against
both Ca$^{2+}$-dependent and non-Ca$^{2+}$-dependent epitopes of Dsg3. The proportion of
antibodies against the Ca$^{2+}$-dependent epitopes decreased in 6 out of the 8 patients. This
result indicated that there were more antibodies against the Ca$^{2+}$-dependent epitopes in
the active phase, and more antibodies against the non-Ca$^{2+}$-dependent epitopes remained
in the inactive phase. In these cases, clinical improvement was observed especially when
antibodies against the Ca$^{2+}$-dependent epitopes decreased. For example, in cases 4 and 5,
conventional Dsg3 ELISA index values were increased in the inactive phase, while
conformational Dsg3 ELISA index values were decreased (Table 1). In case 2, conventional
Dsg3 ELISA index value showed a gradual decline after entering the inactive phase, while
conformational Dsg3 ELISA index value showed a rapid decline (Fig. 3). However, in cases
7 and 8, both conventional and conformational Dsg3 ELISA index values were decreased
(Table 1), while the ratio of antibodies against the Ca$^{2+}$-dependent epitopes increased (Fig.
4). Alternatively, in all cases, conformational Dsg3 ELISA index values were decreased, but
did not show negative in the inactive phase. It is possible that pathogenic antibodies against the Ca^{2+}-dependent epitopes decreased after entering the inactive phase, and nonpathogenic antibodies against the Ca^{2+}-dependent epitopes, such as AK 15 and AK18 mAbs, were detected. EDTA-treated ELISA could distinguish antibodies against Ca^{2+}-dependent epitopes from non-Ca^{2+}-dependent epitopes of Dsg3, but could not distinguish pathogenic from nonpathogenic antibodies against the Ca^{2+}-dependent epitopes.

Another possible explanation is that low-dose prednisolone and/or adjuvant therapy suppressed the disease activity. It is also possible that in mucocutaneous-type PV patients the decrease of anti-Dsg1 serum antibodies led to the clinical improvement.

To evaluate the pathogenicity of PV-IgG associated with EDTA-treated Dsg3 ELISA, we examined the pathogenicity of antibodies against Ca^{2+}-dependent and non-Ca^{2+}-dependent epitopes of Dsg3 using dissociation assay. The ability of baculoproteins to adsorb autoantibodies was restored after EDTA-treated Dsg3 baculoproteins were dialyzed against PBS-Ca. We confirmed that the effect of EDTA on the Ca^{2+}-dependent structure of Dsg3 was reversible, as previously reported. The result of the cell-cell dissociation induced by PV-IgG after immunoadsorption with each Dsg3 baculoprotein suggests that antibodies against the Ca^{2+}-dependent epitopes of Dsg3 mainly contribute to the pathogenicity of
PV-IgG. Moreover, the antibodies against the non-Ca\textsuperscript{2+}-dependent epitopes of Dsg3 were nonpathogenic, and did not enhance the effects on disruption of intercellular adhesion as far as determined by in vitro dissociation assay.

A previous study showed that IgG titers against NH\textsubscript{2}-terminus of Dsg3 (1-161) (Fig. 1) correlated with the disease activity of PV\textsuperscript{24}. In addition to pathogenic antibodies against NH\textsubscript{2}-terminus of Dsg3 (1-161), antibodies against the EC2, EC3 and EC4 may act synergistically in inducing the intraepidermal loss of adhesion and decreased with the improvement of the disease\textsuperscript{25}. Moreover, antibodies against the EC1 and EC5 were detected in the inactive phase, while antibodies against the EC2, EC3 and EC4 were not.

EDTA-treated ELISA can distinguish antibodies against Ca\textsuperscript{2+}-dependent epitopes (i.e. Dsg3 (1-161)) and non-Ca\textsuperscript{2+}-dependent epitopes (i.e. Dsg3 (403-565)). Therefore, EDTA-treated ELISA can be useful for detecting pathogenic anti-Dsg3 serum antibodies more accurately in clinical situations, especially in the inactive phase of PV. When the dose of prednisolone or immunosuppressive drugs is tapered, EDTA-treated ELISA provides a clearer indication of what proportion of anti-Dsg3 serum antibody titer is composed of pathogenic antibodies. Moreover, when a rise in anti-Dsg3 serum antibody titers before a disease relapse was observed, the ratio of antibodies against the Ca\textsuperscript{2+}-dependent epitopes or the
non-Ca\textsuperscript{2+}-dependent epitopes would be useful information to predict the disease activity.

Acknowledgments

We thank Toshio Demitsu for providing the serum samples.
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Figure legends

Fig 1. Molecular structure of Dsg3 and the epitopes recognized by various AK mAbs.

The epitopes recognized by AK mAbs were mapped by immunoprecipitation of domain-swapped Dsg3 recombinant molecules \(^{12}\).

Fig 2. Affinity of each anti-Dsg3 mAb to the 0.5 mM EDTA-treated Dsg3 recombinant protein.

EDTA-treated ELISA index values for AK23 (a) and AK19 (b) mAbs directed against the Ca\(^{2+}\)-dependent conformational epitopes decreased markedly compared to conventional ELISA index values. However, the affinity of AK20 (c) mAb directed against the non-Ca\(^{2+}\)-dependent epitopes was not affected.

Fig 3. Conformational ELISA index values of 8 PV patients’ sera, which remained elevated conventional Dsg3 ELISA index values greater than 100 index values even in the inactive phase.

Anti-Dsg3 ELISA index values as determined using conventional ELISA for 8 patients in the active phase of PV were decreased, but remained high after they entered the inactive
phase. Anti-Dsg3 ELISA index values were increased in 2 patients after entering the inactive phase. On the other hand, calculated conformational ELISA index values were decreased in all patients.

Fig 4. The ratio of anti-Dsg3 serum antibodies recognizing Ca$^{2+}$-dependent and non-Ca$^{2+}$-dependent epitopes in active and inactive phases.

The ratio of antibodies recognizing the Ca$^{2+}$-dependent epitopes decreased in 6 out of the 8 patients after entering the inactive phase.

Fig 5. Dose-dependent effects of purified IgG from PV patients’ sera with different conformational ELISA index values on disruption of intercellular adhesion.

Monolayers of DJM-1 cells were treated with each purified IgG at concentrations between 0.032 and 1000 µg/ml. Purified IgG in case 1, which were obtained in the active phase and contained antibodies against the Ca$^{2+}$-dependent epitopes, showed dose-dependent reactivity between 4 and 1000 µg/ml. Purified IgG in case 2, which did not have pathogenicity clinically and had no antibodies against the Ca$^{2+}$-dependent epitopes, did not induce fragmentations of the cell sheet.
Fig. 6 Effects of PV-IgG against non-Ca^{2+}-dependent epitopes on disruption of intercellular adhesion.

Purified IgG were obtained from 2 PV patients (No. 1, 2 and No. 3, 4). Cell sheets were incubated with each PV-IgG for 8 hours (a) and 24 hours (b). The number of cell fragmentations incubated with PV-IgG No. 1 was nearly equal to that of PV-IgG No. 2. Similarly, the number of cell fragmentations incubated with PV-IgG No. 3 was nearly equal to that of PV-IgG No. 4.

Fig. 7 Alteration of the immunoreactivity of PV-IgG after immunoadsorption with each Dsg3 baculoprotein.

PV-IgG were incubated with each Dsg3 baculoprotein. As the concentrations of untreated Dsg3 baculoproteins and EDTA-treated Dsg3 baculoproteins dialyzed against PBS-Ca increased, the immunoreactivities of PV-IgG were strongly blocked. On the other hand, the immunoreactivity of PV-IgG adsorbed with EDTA-treated Dsg3 baculoproteins reached a plateau level, and that was nearly equal to conformational ELISA index value of PV-IgG.
Fig 8. Blocking effects of various Dsg3 baculoprotein treatments on dissociation assay.

Cell sheets incubated with EDTA-treated Dsg3-adsorbed PV-IgG (b) dissociated into numerous smaller fragments, with the number of fragmentations equal to that of the positive control (a). By contrast, PV-IgG adsorbed with untreated Dsg3 baculoproteins (d) and PV-IgG adsorbed with EDTA-treated Dsg3 baculoproteins dialyzed against PBS-Ca (c) did not cause fragmentations, as well as the negative control (e). The cell fragmentations were quantified as shown in the graph.
Table 1. Antibody profiles of the serum from PV patients with anti-Dsg3 ELISA index value greater than 100 index value in the inactive phase.

<table>
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<th>Patient</th>
<th>Dsg1 ELISA index</th>
<th>Active phase</th>
<th>Conventional ELISA index (A)</th>
<th>EDTA ELISA index (B)</th>
<th>Conformational ELISA index (C)</th>
<th>(C/A x 100)</th>
<th>Dsg3 ELISA index</th>
<th>Conventional ELISA index (A)</th>
<th>EDTA ELISA index (B)</th>
<th>Conformational ELISA index (C)</th>
<th>(C/A x 100)</th>
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<td>1 61yF</td>
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<td>4.9</td>
<td>1182.3</td>
<td>1007.9</td>
<td>174.4</td>
<td>14.8%</td>
<td>2.5</td>
<td>305.2</td>
<td>161.2</td>
<td>144.0</td>
<td>47.2%</td>
<td></td>
</tr>
</tbody>
</table>

A: Conventional ELISA index value.

B: EDTA-treated ELISA index value.

C: Conformational ELISA index value = conventional ELISA index value — EDTA-treated ELISA index value (A – B).
Table 2. Antibody profiles of the serum from PV patients used for \textit{in vitro} dissociation assay.

**a)**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical disease activity</th>
<th>Serum Dsg1 ELISA index</th>
<th>Serum Dsg3 ELISA index</th>
<th>Serum Purified IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(A)</td>
<td>(B)</td>
<td>(C)</td>
</tr>
<tr>
<td>1 59y, F</td>
<td>+</td>
<td>6.1</td>
<td>68.8</td>
<td>3.6</td>
</tr>
<tr>
<td>2 78y, F</td>
<td>-</td>
<td>362.6</td>
<td>67.7</td>
<td>75.8</td>
</tr>
</tbody>
</table>

**b)**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Serum No</th>
<th>Clinical disease activity</th>
<th>Serum Dsg3 ELISA index</th>
<th>Serum Purified IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(A)</td>
<td>(B)</td>
</tr>
<tr>
<td>41y, M</td>
<td>1</td>
<td>+</td>
<td>96.0</td>
<td>63.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>43.3</td>
<td>14.0</td>
</tr>
<tr>
<td>80y, F</td>
<td>3</td>
<td>-</td>
<td>46.1</td>
<td>36.8</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>14.2</td>
<td>8.6</td>
</tr>
</tbody>
</table>
Fig 1
Fig 2

Graphs (a) through (e) show the concentration of various substances plotted against absorbance or other measurements. Each graph compares conventional ELISA and EDTA-treated ELISA methods.

- (a) Concentration of AK23 (μg·mL⁻¹) vs. absorbance.
- (b) Concentration of AK13 (μg·mL⁻¹) vs. absorbance.
- (c) Concentration of AK18 (μg·mL⁻¹) vs. absorbance.
- (d) Concentration of AK18 (μg·mL⁻¹) vs. absorbance.
- (e) Concentration of AK20 (μg·mL⁻¹) vs. absorbance.
Fig 3
Fig 5
Fig 7
Fig 8

a) Anti-Dsg3 serum-IgG

b) Anti-Dsg3 serum-IgG adsorbed with EDTA-treated Dsg3 baculoproteins

c) Anti-Dsg3 serum-IgG adsorbed with EDTA-treated Dsg3 baculoproteins dialyzed against PBS-Ca

d) Anti-Dsg3 serum-IgG adsorbed with untreated Dsg3 baculoproteins

e) No anti-Dsg3 serum-IgG

![Graph of experimental results](image)