Pemphigus Vulgaris-IgG Causes a Rapid Depletion of Desmoglein 3 (Dsg3) from the Triton X-100 Soluble Pools, Leading to the Formation of Dsg3-Depleted Desmosomes in a Human Squamous Carcinoma Cell Line, DJM-1 Cells

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In this study, we examined desmoglein (Dsg) 3 and other desmosomal molecules after pemphigus vulgaris (PV)-immunoglobulin G (IgG) binding to the Dsg3 on the cell surface in DJM-1 cells, a human squamous cell carcinoma cell line. After cells were incubated with PV-IgG for various time periods (0, 5, 10, 20, 30, 60 min, or 30 h), cells were fractionated into phosphate-buffered saline soluble (cytosol), phosphate-buffered saline insoluble-Triton X-100 soluble (membrane), and Triton X-100 insoluble (cytoskeleton) fractions, and subjected to immunoblotting and immunofluorescence microscopy using antibodies against Dsg1, Dsg3, plakoglobin, desmoplakin 1, and cytokeratins. Immunoblot analysis with PV-IgG revealed that Dsg3 was already dramatically depleted from the membrane fraction 20 min after PV-IgG treatment, whereas no reduction of Dsg3 was detected in the cytoskeleton fraction as examined by immunoblotting. A 30 h incubation with PV-IgG, however, caused a marked disappearance of Dsg3, but not other desmosomal molecules, from cytoskeleton fractions. Furthermore, double-staining immunofluorescence microscopy revealed that Dsg3 was depleted from the desmosomes whereas Dsg1, desmoplakin 1, plakoglobin, and keratin filaments were bound to desmosomes. These results provide a novel interpretation for a better understanding of mechanisms for blistering in PV; i.e., a possibility that PV-IgG generates the formation of aberrant desmosomes, which are lacking in Dsg3, but not other desmosomal constituents. ***Key words: autoimmune blistering disease/desmoplakin/keratin intermediate filament/plakoglobin. J Invest Dermatol 112:67–71, 1999***

Pemphigus vulgaris (PV) and pemphigus foliaceus are autoimmune skin diseases in which loss of adhesion between keratinocytes (i.e., acantholysis) appears to be mediated by binding of autoantibodies against desmoglein (Dsg) 3 (Eyre and Stanley, 1988; Hashimoto *et al*, 1990; Buxton *et al*, 1993) and Dsg1 (Koulu *et al*, 1984; Andrew *et al*, 1995), respectively. These target molecules of autoantibodies, Dsg3 and Dsg1, are core proteins of desmosome (Eyre and Stanley, 1988; Karpati *et al*, 1993), which form a 210 kDa or a 240 kDa protein complex by association with plakoglobin (PG), respectively (Korman *et al*, 1985). Although it is now widely accepted that autoantibodies against Dsg3 and Dsg1 are the primary pathogenic agents (Amagai, 1995), the key question concerning the exact mechanism for acantholysis after PV- or pemphigus foliaceus-immunoglobulin G (IgG) binding to the antigens on the cell surface has not yet been elucidated. Pemphigus-induced acantholysis may be caused either by proteinases from keratinocytes (Morioka *et al*, 1981), such as plasminogen activator (Hashimoto *et al*, 1983), or possibly by direct binding of PV-IgG to desmosomal molecules to disrupt cell–cell attachment (Koch *et al*, 1997). We have previously demonstrated that the Ca^{++}-induced desmosome formation generated by a change from low (<0.09 mM) to high (>1.0 mM) Ca^{++} concentration, was not inhibited by PV-IgG binding to the surface PV antigen. Desmosomal bridges, however, which were formed following the change in Ca^{++} concentration, in the presence of PV-IgG, were dissociated within 24–36 h after PV-IgG binding to the cell surface (Kitajima *et al*, 1987). This time lag of 24–36 h to produce cell–cell detachment suggests that any cell biologic response leading to cell detachment may be involved, rather than be a direct inhibition of desmosome formation.

From these viewpoints, we have recently studied the effects of PV-IgG on the intracellular signal transduction events and demonstrated that PV-IgG binding to DJM-1 cells (a squamous cell carcinoma line) induces a transient increase in activation of phospholipase C (Esaki *et al*, 1995) and increased inositol 1,4,5-trisphosphate production, with a concomitant increase in intracellular calcium concentration ($[Ca^{++}]i$) (Seishima *et al*, 1995), and characteristic activation of diverse isomers of protein kinase C, a family of Ca^{++}- and phospholipid-dependent serine/threonine kinase (Osada *et al*, 1997), linked to secretion of plasminogen activators and expression of plasminogen activator receptor (Seishima *et al*, 1997; reviewed in Kitajima, 1996).

In this study, we determined what happened to the desmosomal constituents, Dsg1, Dsg3, PG, and desmoplakin (Dpk) 1, during cell detachment induced by PV-IgG in cultured human transformed keratinocytes, DJM-1 cells, by immunofluorescence microscopy

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Abbreviations: Dpk, desmoplakin; Dsg, desmoglein; IgG, immunoglobulin G; KIF, keratin intermediate filament; PG, plakoglobin; PV, pemphigus vulgaris.

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and immunoblotting using antibodies against these desmosomal constituents. The reason DJM-1 cells are used as an experimental cell system for PV-IgG-cell detachment, is that this cell line has been shown to be much more sensitive to the PV-IgG-induced cell–cell detachment and the number of desmosomes has been shown to be much smaller than with normal keratinocytes, so that the effects of PV-IgG on desmosomes can be clearly detected in morphology. Immunoblotting revealed that Dsg3 had already disappeared from a pool, i.e., the phosphate-buffered saline (PBS) insoluble-Triton X-100 soluble (membrane) fraction, before being integrated into desmosomes 20 min after the addition of PV-IgG in the culture medium, whereas Dsg3 associated with the Triton X-100 insoluble (cytoskeleton) fraction had not. Surprisingly, however, after 30 h incubation, Dsg3 was almost completely deleted from cytoskeleton fractions, and this deletion was seen in desmosomes as illustrated by immunofluorescence microscopy. These data tempted us to speculate that PV-IgG may generate an impaired epidermis, the desmosomes of which have abnormally low Dsg3 levels.

MATERIALS AND METHODS

Antibodies Monoclonal antibody (PG5.1) against PG and monoclonal antibody (DP2.17) against Dpk1 were obtained from Progen (Heidelberg, Germany). Monoclonal antibody against Dsg1/2 and that against keratin 8 were obtained from Progen and DAKO (Carpinteria, CA), respectively.

Purification of IgG Sera from six patients with PV, and normal volunteers without any skin disease, were used for these experiments. All sera were heated to 56°C for 30 min to inactivate complement, and were stored at –80°C. IgG fractions were isolated from these sera by using Mab Trap GII kit (Pharmacia AB, Uppsala, Sweden). The purity of isolated IgG was checked by electrophoresis.

Cell cultures An isolated cell line (DJM-1) from human skin squarnous cell carcinoma was used in this study (Kitajima *et al*, 1987, 1988). Cells were cultured in minimum essential medium (Life Technologies, Grand Island, NY) containing 10% fetal calf serum, 0.4 mg hydrocortisone per ml, 10 mg epidermal growth factor per ml, 84 ng cholera toxin per ml, 100 mg kanamycin per ml, and 1.8 mM Ca^{2+}. At 80%–90% confluence, cells were exposed to PBS containing 0.25% trypsin and 10 mM ethylenediamine tetraacetic acid for 10 min at 37°C. They were resuspended in minimum essential medium with 10% fetal calf serum, cultured for 2 d, and used as described below.

Treatment with PV-IgG and cell fractionation To determine what happens to desmosomal constituents, cells were fractionated into PBS soluble (cytosol), PBS insoluble-Triton X-100 soluble (membrane), and Triton X-100 insoluble (cytoskeleton) fractions. After cells reached confluence, PV-IgG or normal IgG was directly added into the culture medium at a final concentration of 0.3 mg per ml and cells were incubated for 0, 5, 10, 20, 30, 60 min, or 30 h. Because it was confirmed that complement-inactivated sera (treated with 56°C for 30 min) exerted the same results as purified IgG, repeating experiments were carried out with PV sera. Cells were then washed twice with PBS and harvested with buffer A containing 1 mM phenylmethyisulfonyl fluoride (Sigma, St. Louis, MO) and 5 mM E64 (Peptide Institute, Osaka, Japan) in PBS. After sonication (five times for 5 s for each), cell homogenates were centrifuged at 105,000 × *g* for 60 min. The supernatant was stored as the cytosol fraction at –80°C. The pellet was solubilized with buffer A containing 1% Triton X-100 and centrifuged at 105,000 × *g* for 60 min. The resultant supernatant was stored as the membrane fraction. The Triton X-100 insoluble pellet was solubilized with 0.25 M Tris, pH 6.8, 2% sodium dodecyl sulfate, 30% glycerol, and 10% β-mercaptoethanol, and stored as the cytoskeleton fraction at –80°C. Protein concentrations were determined spectrophotometrically using the Bio-Rad with protein assay kit (Bio-Rad, Richmond, CA).

Immunoblotting Aliquots of the cytosol, membrane, and cytoskeleton fractions were mixed with sample buffer (10% 2-β-mereaptoethanol, 2% sodium dodecyl sulfate, 30% glycerol, 0.25 M Tris, pH 6.8, final concentration), and heated at 98°C for 5 min. Samples of each fraction (30 μg of protein per lane) were subjected to reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis 10% polyacrylamide gels by the method of Laemmli (1970). Protein standards for molecular weight (Bio-Rad) were as follows: ovalbumin, 49 kDa; bovine serum albumin, 80 kDa; P-galactosidase, 116.5 kDa; and myosin, 205 kDa. Keratin 8 was used as an internal standard to analyze semiquantitatively the desmosomal constituents.

Immunofluorescence microscopy The cells cultured on the glass coverslips were treated with PV-IgG or complement-inactivated sera obtained from patients with PV or normal individuals for 20 min or 30 h.

The cells, on the coverslips, were rinsed with ice cold PBS and fixed by dipping for 10 min in –20°C methanol, followed by incubation and washing with PBS containing 0.05% Triton X-100. The fixed cells were first incubated with PV sera overnight and stained with anti-human IgG labeled rhodamine for 45 min after washing with PBS, and then incubated for 2 h with one of the monoclonal antibodies directed to Dsg I, PG, Dpk1, and cytokeratins, followed by staining with anti-mouse IgG labeled with fluorescent isotiocloride (Cappel, Durham, NC) for 45 min. Controls for specificity of the antibodies were routinely carried out. Photomicrographs were taken with a Nikon FX-RFD photomicroscope (Nikon, Tokyo, Japan).

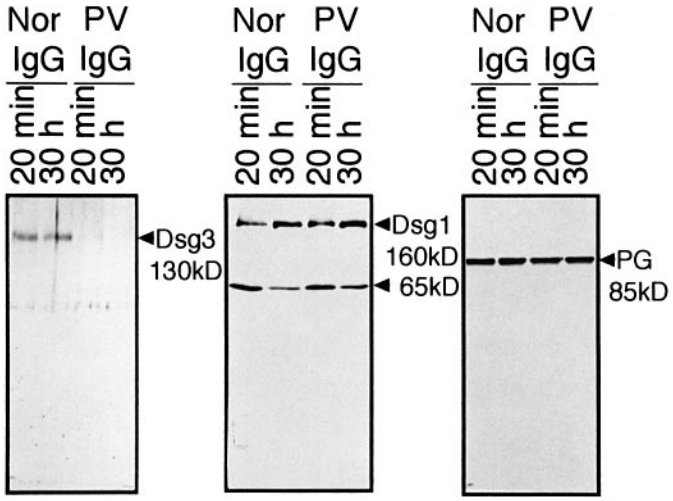


Figure 1. Immunoblots of membrane (PBS insoluble-Triton X-100 soluble) fractions obtained after 20 min or 30 h stimulation with PV-IgG (PV) or normal human IgG (Nor) in DJM-1 cells, using anti-Dsg1, Dsg3, and plakoglobin (PG) antibodies. Dsg 3 was depleted almost completely from membrane fractions 20 min and also 30 h after PV-IgG stimulation, whereas normal IgG stimulation caused no depletion of Dsg3. In contrast, Dsg1 and PG were not depleted at any time after PV-IgG stimulation as well as normal IgG stimulation.

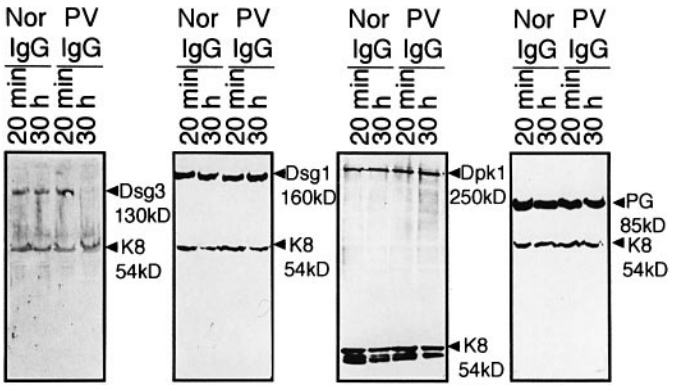


Figure 2. Immunoblots of cytoskeleton (Triton X-100 insoluble) fractions obtained after 20 min or 30 h stimulation with PV-IgG (PV) or normal human IgG (Nor) in DJM-1 cells, using anti-Dsg1, Dsg3, Dpk1, PG, and keratin 8 (K8). Stimulation with PV-IgG for 30 h caused almost complete depletion of Dsg 3 from cytoskeleton fractions, whereas Dsg1, Dpk1, and PG were not deleted by this stimulation. Note that stimulation with PV-IgG for 20 min is not enough to cause depletion of Dsg3, as is clearly demonstrated by comparing the densities of bands of keratin 8 and Dsg3. Keratin 8 (K8) is used for a quantitative control.

RESULTS

PV-IgG caused a depletion of Dsg3 from its pool and cytoskeleton fractions as detected by immunoblotting Dsg1, Dsg3, Dpk1, and PG were measured after cells were treated with PV-IgG for 0, 5, 10, 20, 30, 60 min, and 30 h, because our previous studies showed that phosphorylation of Dsg3 was caused after 20 min treatment and cell–cell detachment was generated after 30 h treatment with PV-IgG in DJM-1 cells. After cell fractionation, aliquots of PBS soluble (cytosol), PBS insoluble-Triton X-100 soluble (membrane), and Triton X-100 insoluble (cytoskeleton) fractions were processed for immunoblotting with antibodies against Dsg1, Dpk1, PG, and with PV-IgG for detection of Dsg3. Immunoblots of cytoskeleton fractions were subjected to double-staining with one of these antibodies and monoclonal antibody to keratin 8 as an internal standard for semiquantitative analysis.

The results showed that a marked reduction or an almost complete disappearance of Dsg3, but not of Dsg1 or PG, from the membrane fraction was seen 20–60 min and 30 h, although not at 0–10 min, after treatment with PV-IgG, whereas treatment with normal IgG did not affect the amount of any of Dsg3, Dsg1, and PG (**Fig 1**). In contrast to membrane fractions, no detectable reduction of Dsg3, as well as of Dpk1 and PG, from the cytoskeleton fractions was caused 20–60 min after treatment with PV-IgG. A 30 h treatment with PV-IgG, however, deleted Dsg3 almost completely from the cytoskeleton fractions, whereas again it had no affect on Dsg1, PG, and Dpk1. The reduction in the quantity of Dsg3 was confirmed by comparing with the amount of keratin 8 as an internal standard after PV-IgG treatment (**Fig 2**). Normal IgG also exerted no effects on any of Dsg1, Dsg3, PG, and Dpk1 in cytoskeleton fractions (**Fig 2**). Taken together, these results imply that PV-IgG treatment substantially reduced the amount of Dsg3 from the Triton X-100 soluble (membranes) pool early in the experiment, i.e., after 20 min, and from the Triton X-100 insoluble (cytoskeletons) pool later in the experiment, i.e., after 30 h.

PV-IgG deleted Dsg3 from desmosomes as revealed by immunofluorescene Because only Dsg3, and not other desmosomal molecules, was depleted from cytoskeleton fractions 30 h after the treatment with PV-IgG as revealed by immunoblotting, we then determined whether this was morphologically observed using immunofluorescence microscopy. After treated with 1% Triton X-100, the PV-IgG stimulated cells cultured on the glass coverslips were double-stained with anti-Dsg3 antibody (PV-IgG) and one of the monoclonal antibodies against Dsg1, PG, Dpk1, and cytokeratin 8.

Double-immunofluorescence analysis using anti-Dsg3 (PV-IgG) and monoclonal antibody-directed cytokeratin 8 showed that, in the cells stimulated with PV-IgG for 20 min, Dsg3 was localized, forming a punctate line at cell–cell contacts to which bundles of keratin intermediate filaments (KIF) emanating from the juxta nuclear region were bound, illustrating a radial organization in the cell (**Fig 3**). In cells double-stained with anti-Dsg3 and one of anti-Dsg1, anti-Dpk1, and anti-PG antibodies, the proteins were colocalized along the plasma membrane generating a punctate or linear line, whereas staining of PG revealed a more linear rather than punctate line (**Fig 3**). These findings were the same as observed in control cells treated with normal IgG for 20 min (not shown). It should be noted that Dsg3 was found to be colocalized with any of these desmosomal proteins as expected by immunoblotting analysis, which showed that Dsg3 was not depleted from the cytoskeleton, but was deleted from membrane fractions 20 min after PV-IgG stimulation as shown by immunoblotting.

In contrast, in most of the cells incubated with PV-IgG for 30 h, double-staining with anti-Dsg3 and anti-cytokeratin antibodies revealed that Dsg3 disappeared dramatically from the cell–cell contacts, which were still retained as shown by anti-keratin staining, in the peripheral cells of the colony. This was less prominent in the center of the colony (**Fig 4*c*, *d***). The disappearance of Dsg3 was more clearly demonstrated by double-label immunofluorescence staining using anti-Dpk1 and Dsg3. Even after 30 h stimulation with PV-IgG, Dpk1 maintained a punctate linear distribution pattern at cell–cell contacts, whereas Dsg3 disappeared dramatically from many of the cell–cell contacts (**Fig 4*k*, *l***). The same results were obtained in cells stained by double-label immunofluorescence with anti-Dsg3 and any of the monoclonal antibodies directed to Dsg1 and PG (**Fig 4*g*, *h*, *o*, *p***). In cells treated with normal human IgG, Dsg3 remained in a linear arrangement along cell–cell contacts (**Fig 4*b*, *f*, *j*, *n***). All these results demonstrate that 30 h stimulation with PV-IgG markedly deleted Dsg3, but not other desmosomal components, i.e., Dsg1, PG, and Dpk1, from desmosomes, to which KIF were still bound.

DISCUSSION

An intriguing finding in this study is that the PV-IgG binding to Dsg3 on the cell surface caused a marked reduction of only Dsg3, and not Dsg1, Dpk1, and PG, from the membrane (Triton X-100 soluble) fraction early on, i.e., after 20 min, and also from the

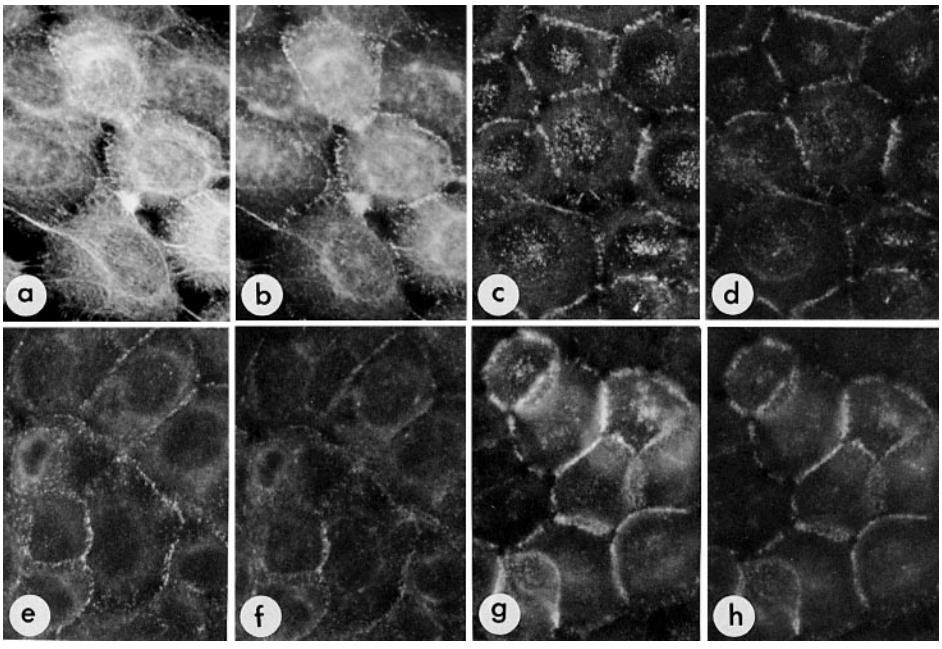


Figure 3. Double-staining immunofluorescence microscopy of DJM-1 cells stimulated with PV-IgG for 20 min using anti-keratin 8 (*a*), anti-Dsg1 (*c*), anti-Dpk1 (*e*), anti-PG (*g*), and Dsg3 (*b*, *d*, *f*, *h*) antibodies. Cells were fixed with cold methanol (–20°C) for 10 min. Dsg3 are colocalized with any of Dsg1, Dpk1, and PG.

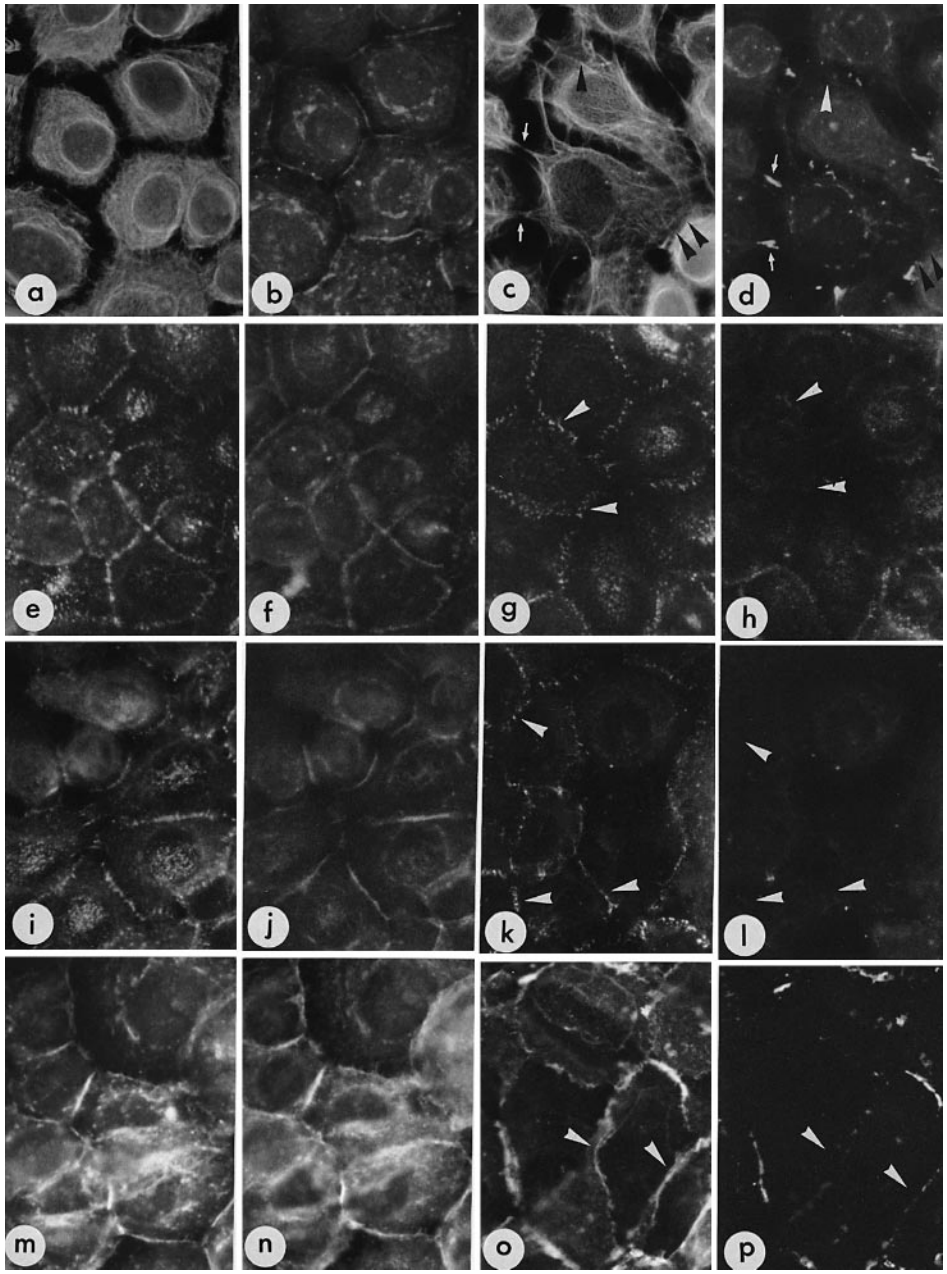


Figure 4. Double-staining immunofluorescence microscopy of DJM-1 cells stimulated with PV-IgG (*c*, *d*, *g*, *h*, *k*, *l*, *o*, *p*) or normal IgG (*a*, *b*, *e*, *f*, *i*, *j*, *m*, *n*) for 30 h using anti-keratin 8 (*a*, *c*), anti-Dsg1 (*e*, *g*), anti-Dpk1 (*i*, *k*), anti-PG (*m*, *o*), and Dsg3 (*b*, *d*, *f*, *h*, *i*, *l*, *n*, *p*) antibodies. Cells were fixed with cold methanol (–20°C) for 10 min. Note that the number of immunofluorescent points of Dsg3 was dramatically reduced from the majority of points (desmosomes) where keratin intermediate filaments are connected point-to-point with each other 30 h after stimulation with PV-IgG (*c*, *d*), whereas Dsg3 is clearly detected at cell–cell contacts, forming a linear fluorescence 30 h after normal IgG stimulation (*a*, *b*). *Small arrows* (*d*) indicate remnants of Dsg3 at desmosornes, whereas *arrowheads* indicate the points where no Dsg3 is detected at joints of bundles of keratin intermediate filaments (*c*, *d*). Dsg3 (*h*, *l*, *p*; *arrowheads*) is dramatically deleted from desmosomes where Dsg1 (*g*; *arrowheads*), Dpk1 (*k*; *arrowheads*), and PG (*p*; *arrowheads*) are clearly detected in samples obtained 30 h after PV-IgG stimulation. A deletion or a marked reduction in the fluorescence intensity of Dsg3 (*d*, *h*, *l*, *p*) was also clearly demonstrated 30 h after PV-IgG stimulation, as compared with cells treated with normal IgG (*b*, *f*, *j*, *n*).

cytoskeleton (Triton X-100 insoluble) fraction, which includes desmosomes, later on, i.e., 30 h after stimulation with PV-IgG, as revealed by immunoblotting analysis.

Because the depletion of Dsg3 from the Triton X-100 insoluble cytoskeleton fraction was generated 30 h after the earlier (20–60 min) deletion of Dsg3 from a Triton X-100 soluble pool after PV-IgG stimulation, the depletion of Dsg3 from the cytoskeleton fraction may be caused by a shortage of the Dsg3 pool for its integration into desmosomes.

It is also of great interest to note that the depletion of Dsg3 from the Triton X-100 insoluble (cytoskeletons including desmosomes) fraction revealed by immunoblotting turned out to be that from desmosomes, when determined by double-staining immunofluorescence microscopy using anti-Dsg3 antibodies and one of the monoclonal antibodies against desmosomal molecules and keratin 8. It was also consistent with immunoblotting analysis that the PV-IgG-induced depletion of desmosomal constituents was limited to Dsg3, whereas other components, i.e., Dsg1, Dpk1, and PG, were retained to be intact in desmosomes even 30 h after PV-IgG stimulation by immunofluorescence analysis. Another surprising finding was that, even though almost all Dsg3 was depleted from desmosomes in the peripheral cells of the colony, KIF were still connected to desmosomes as revealed by double-staining with monoclonal anti-keratin antibody. The Dsg3-depleted desmosomes appear to maintain the integrity well enough to keep the cell–cell contact associated with KIF at least in culture.

The fact that the depletion of Dsg3 from desmosomes was prominent in the periphery of the colony, whereas it was not always observed in the center of the colonies, may be due to the shorter turnover time of desmosomes, which are more likely to take place in the periphery of the colony, where cell migration and proliferation are higher in activity, than in the center of the colony. The turnover time in the periphery of the colony is supposed to be short enough to visualize a formation of the Dsg-3 deleted desmosomes because of the shortage of Dsg3 after 30 h PV-IgG stimulation. The reason Dsg3 was found at desmosomes 20 min after PV-IgG stimulation, is that 20 min may not be long enough to replace the old pre-existing desmosomes with newly formed Dsg3-deleted desmosomes.

Considering the function of Dsg3, i.e., binding to PG and KIF, it is of interest to note that the deletion of Dsg3 did not particularly affect the KIF binding to desmosomes. This suggests that other

components compensate for KIF binding to desmosomes and desmosomal integrity to a certain extent. The cell–cell adhesion by Dsg3-deleted desmosomes, however, may not to be strong enough to maintain the normal strength of the epidermis, and may be a key cause for blistering in PV, as Dsg3-knockout mouse showed a phenotype with blistering along the suprabasal layer, similar to PV (Koch *et al*, 1997). Even if depletion of Dsg3 is incomplete, it may be pathogenic as seen in transgenic mice with N-terminal truncated Dsg3 gene, which generated widening of intercellular spaces, a reduction of the number of desmosomes, and aberrant desmosomes (Allen *et al*, 1996).

Expression of the desmoglein isoforms correlates well with epidermal differentiation. It has been experimentally shown that Dsg1 is strongly expressed in the upper layers of the epidermis, whereas Dsg3 is predominantly expressed in the lower layers of the epidermis, and Dsg2 is mainly expressed in the basal cells (Koch *et al*, 1992; Arnemann *et al*, 1993; Shimizu *et al*, 1995; Amagai *et al*, 1996; Schaefer *et al*, 1996). Regarding the function of Dsg3, the expression of which is limited to the lower epidermis, it is very interesting to note that Dsg3-knockout mouse showed a phenotype with blistering along the suprabasal layer as seen in PV (Koch *et al*, 1997). These findings suggest that Dsg3 is required to maintain structurally and functionally stable desmosomes. Taken together, it may be reasonably speculated that the PV-IgG-induced depletion of Dsg3 from desmosomes, as found in the present experimental cell system, may be generated in the epidermis, and may also represent a major cause of acantholysis in the lower epidermis of PV patients. It is also intriguing to speculate that the failure of PV-IgG to deplete Dsg1 from desmosomes is the most probable explanation for the clinical features of PV. PV is characterized by acantholyisis in the lower epidermis, but not in the upper epidermis where Dsg1 expression is predominant.

In closing, our results showing that PV-IgG depleted only Dsg3, and not other constituents, from desmosomes in cultured human squamous cell carcinoma cell line cells, imply that PV-IgG-induced depletion of Dsg3 from desmosomes may lead to cell–cell detachment in the epidermis, especially in suprabasal layers where the Dsg1/Dsg3 ratio is small.

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