

# Neutrophils Contact to Plasma Membrane of Keratinocytes Including Desmosomal Structures in Canine Pemphigus Foliaceus

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**ABSTRACT.** Pemphigus foliaceus (PF) is an autoimmune blistering skin disease that affects certain mammals including dogs. In canine PF, neutrophils are infiltrated intensely into pustular lesions including acantholytic cells, although neutrophilic infiltration is not characterized in human PF. The roles of the neutrophils in the cutaneous lesions of canine PF have not yet been understood. The purpose of this study was to characterize the ultrastructural features underlying the acantholysis with pustule formation in canine PF. Four dogs diagnosed as PF on the basis of clinical signs, histopathological findings, and direct and indirect immunofluorescence examinations were performed. Electron microscopy revealed that the acantholytic cells were adjacent to multiple neutrophils in the pustules. At the contact points between neutrophils and acantholytic keratinocytes, half-desmosomes of acantholytic keratinocytes with intact attachment plaques were observed within invaginations of neutrophils. Furthermore, on the surface of acantholytic cells in the pustules, neutrophil granules seemed to be secreted to the surface of acantholytic cells and to degenerate the half-desmosome structures. Neutrophils were also observed within the epidermis adjacent to the pustule. At the intercellular gap between two dissociated keratinocytes, neutrophils inserted its pseudopodia into the gap between the two half-desmosomes of keratinocytes. These findings taken together suggested that, at least in the areas where we analyzed ultrastructurally, neutrophils contact desmosomal structures and seem to play some parts in separation of keratinocytes and degeneration of split-desmosomes in pustules of dogs with PF.

**KEY WORDS:** canine, desmosome, dog, pemphigus, ultrastructure.

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Pemphigus foliaceus (PF) is an autoimmune blistering skin disease that affects human beings and animals, including dogs. In human PF, circulating IgG autoantibodies target a desmosomal cadherin, desmoglein 1 (Dsg1), and cause skin blisters [16]. The histopathological findings of human PF are characterized by intraepidermal blister formation with acantholytic cells and dyskeratotic cells at the superficial layer of the epidermis.

Canine PF is one of the most common autoimmune skin diseases affecting dogs, and its histopathological features are similar to those of human PF, except for the infiltration of numerous neutrophils and/or fewer eosinophils into intraepidermal clefts [7, 11]. Circulating autoantibodies against keratinocyte cell surfaces were recognized in dogs with PF, as determined by indirect immunofluorescence (IIF) examination on cryosections of normal epithelia or cultured canine keratinocytes [6, 9]. Recent paper described that injection of serum IgG from three dogs with PF to neonatal mice caused acantholytic blisters at the superficial layer of epidermis [12]. Early immunoblotting studies have revealed that serum IgG found in dogs with PF recognizes a 148-kDa or 160-kDa antigen in canine keratinocyte extracts,

which is presumed to be a canine homologue of human Dsg1 [10, 17]. On the other hand, a recent paper has demonstrated that serum IgG against the extracellular region of canine Dsg1 was recognized only in some dogs with PF [13]. Thus, the target molecules for pathogenic IgG autoantibodies in majority of dogs with PF have currently been unidentified. Furthermore, although granulocytes are known to infiltrate intensely into the lesional skin of dogs with PF, the exact roles of the granulocytes in development of cutaneous lesions in canine PF have been poorly understood.

Electron micrographs were suspected to provide some information on the mechanisms of acantholysis as well as the role of granulocytes in the pathogenesis of cutaneous lesions in canine PF. Therefore, in this study, we investigated the ultrastructural features of pustules containing acantholytic keratinocytes and granulocytes in dogs with PF using transmission electron microscopic analysis.

## MATERIALS AND METHODS

**Dogs:** Four dogs which were compatible with clinical, histopathological and immunohistochemical findings of canine PF were used in this study (Table 1). Case No. 1 had crusted lesions on the face and scrotum, whereas case Nos. 2–4 had generalized skin lesions consisted of papules, pustules and erosions covered with yellowish to brownish

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Table 1. Signalment, clinical findings, the results of immunofluorescence tests and the findings of electron microscopy (EM) in dogs with pemphigus foliaceus

Case No.	Breed	Sex	Age (years)	Distribution of lesions	DIF <sup>a)</sup>	IIF <sup>b)</sup>	EM findings		
							(i)	(ii)	(iii)
1	Akita	Male	8	Head	+	+	+	+	+
2	Mixed	Female	5	Generalized	+	+	+	–	–
3	Mixed	Male	4	Generalized	+	+	+	–	–
4	Mixed	Male	9	Generalized	+	+	+	+	–

a) DIF: direct immunofluorescence (IgG).

b) IIF: indirect immunofluorescence (IgG).

EM findings:

(i) Half-desmosomes of acantholytic cells within invaginations of neutrophils.

(ii) Elongation of neutrophil cytoplasm between desmosome of 2 keratinocytes.

(iii) Neutrophil granules in the intercellular spaces.

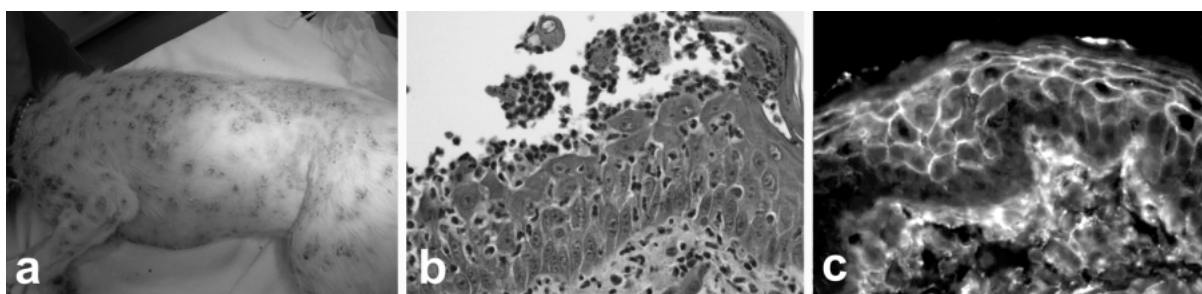


Fig. 1. Clinical photograph of dog#2. Generalized papules, pustules and crusts are observed (Fig. 1a). Photomicrograph of pustular lesion of dog#2. Intraepidermal cleft with acantholysis is observed in the granular layer of epidermis. Several acantholytic cells (arrows) are surrounded by neutrophils. Hematoxylin and eosin. Bar=40  $\mu$ m (Fig. 1b). Direct immunofluorescence of dog#2. The deposition of IgG is recognized on the cell surface of keratinocytes in throughout of the epidermis except for basal layer.  $\times 40$  (Fig. 1c).

crusts (Fig. 1a). None of the four dogs had mucosal lesions despite the extensive skin lesions. Histopathological examination of the skin lesions revealed the intraepidermal pustules containing numerous acantholytic keratinocytes and neutrophils in granular layer of the epidermis (Fig. 1b). These histopathological findings were consistent with features in canine PF.

**Immunofluorescence tests:** To determine the deposition of immunoglobulin on the surface of keratinocytes *in vivo*, direct immunofluorescence (DIF) was performed. Cryosections six  $\mu$ m in thickness of patients' skin were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-dog IgG (1:50, MP Biomedicals, Aurora, OH, U.S.A.), IgA (1:20), IgM (1:20) and C3 (1:20) (Bethyl Laboratories, Montgomery, TX, U.S.A.) antibodies for 30 min at room temperature. After washing with PBS, the sections were observed by fluorescence microscopy (Olympas, Tokyo, Japan).

To detect circulating antibodies against the surface of keratinocytes in dogs with PF, indirect immunofluorescence (IIF) was performed. Cryosections six  $\mu$ m in thickness of the nasal speculum skin obtained from a healthy dog under a generalized anesthesia were incubated for 30 min, respectively with the canine PF sera and 2 normal dog sera, followed by incubation with FITC-conjugated antibodies as the same dilution with DIF for 30 min. After washing with PBS, the sections were observed by fluorescence micro-

copy.

**Transmission electron microscopy:** Skin samples were obtained from a crusted lesion on the nose of case No.1 and from small pustules on the abdomen of case Nos. 2–4 by 6 mm punch biopsies under sedation and local anesthesia. The skin samples were divided into 1 mm<sup>3</sup> pieces and fixed with 2% glutaraldehyde and 1% osmium tetroxide, then dehydrated with graded alcohol and embedded in Epon 812 resin (Okensoyji, Tokyo, Japan). The resin was polymerized at 55°C for 72 hr. Semithin (1  $\mu$ m) sections were stained with 0.1% toluidine blue, and observed under a light microscope prior to the electron microscopic analysis. For analyzing the ultrastructural changes of the early lesions in canine PF, we selected small intraepidermal microscopic pustules, which circumvent gross lesions and contain small number of neutrophils. Ultrathin sections were stained with uranyl acetate and lead citrate, and then examined under a JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan).

## RESULTS

**Immunofluorescence results:** DIF examination revealed the net-like deposition of IgG (Fig. 1c), but not that of IgA, IgM or C3 (date not shown), on the surface of keratinocytes in the perilesional skin in all four cases. In addition, serum IgG against the surface of canine keratinocytes was detected

in all four canine cases by IIF examination using normal canine nasal skin as the substrate. The results of DIF and IIF are summarized in Table 1.

**Ultrastructural findings:** Ultrastructural changes of pustules from four dogs with PF were examined using transmission electron microscopy. Light microscopic analysis of the semithin sections, which was performed prior to electron microscopic analysis, revealed some intraepidermal pustules containing acantholytic keratinocytes in the upper part of epidermis. Neutrophils were recognized both in the intraepidermal pustules and superficial dermis. In pustular lesions, a part of neutrophils were seemed to be crusted around acantholytic keratinocytes (date not shown). Despite the intense infiltration of neutrophils, eosinophils were unremarkable in the lesional skin of all four cases included in this study.

Ultrastructural analysis revealed that the acantholytic cells were adjacent to multiple neutrophils in the pustules (Fig. 2a). Many of the acantholytic keratinocytes had intact nucleus and numerous intracytoplasmic vacuoles, suggestive of the degenerative changes in keratinocytes. At the

contact points between neutrophils and acantholytic cells, half-desmosomes were observed within invaginations of neutrophils under higher magnification (Fig. 2b). Moreover, all of the half-desmosome structures were recognized to have intact attachment plaques and attach tonofilaments (Fig. 2b-e). When we analyzed more than 20 half-desmosomes on the surface of acantholytic keratinocytes in every case, all half-desmosomes were observed at the contact points between neutrophils and acantholytic keratinocytes. On the other hand, the half-desmosome structures were not observed on the keratinocyte cell surfaces wherein neutrophils were not attached.

To examine the ultrastructural changes during an early phase of acantholysis, we focused on a microscopic skin lesion wherein an acantholytic keratinocyte was mostly separated but partially attached to the adjacent keratinocytes in a case No. 1 (Fig. 3a). Neutrophils, which were attached to the surface of acantholytic keratinocytes, extended its cytoplasm toward the cell-cell contact sites between keratinocytes (Fig. 3b). At higher magnification, the neutrophil was found to insert its pseudopodia between adjacent kerat-

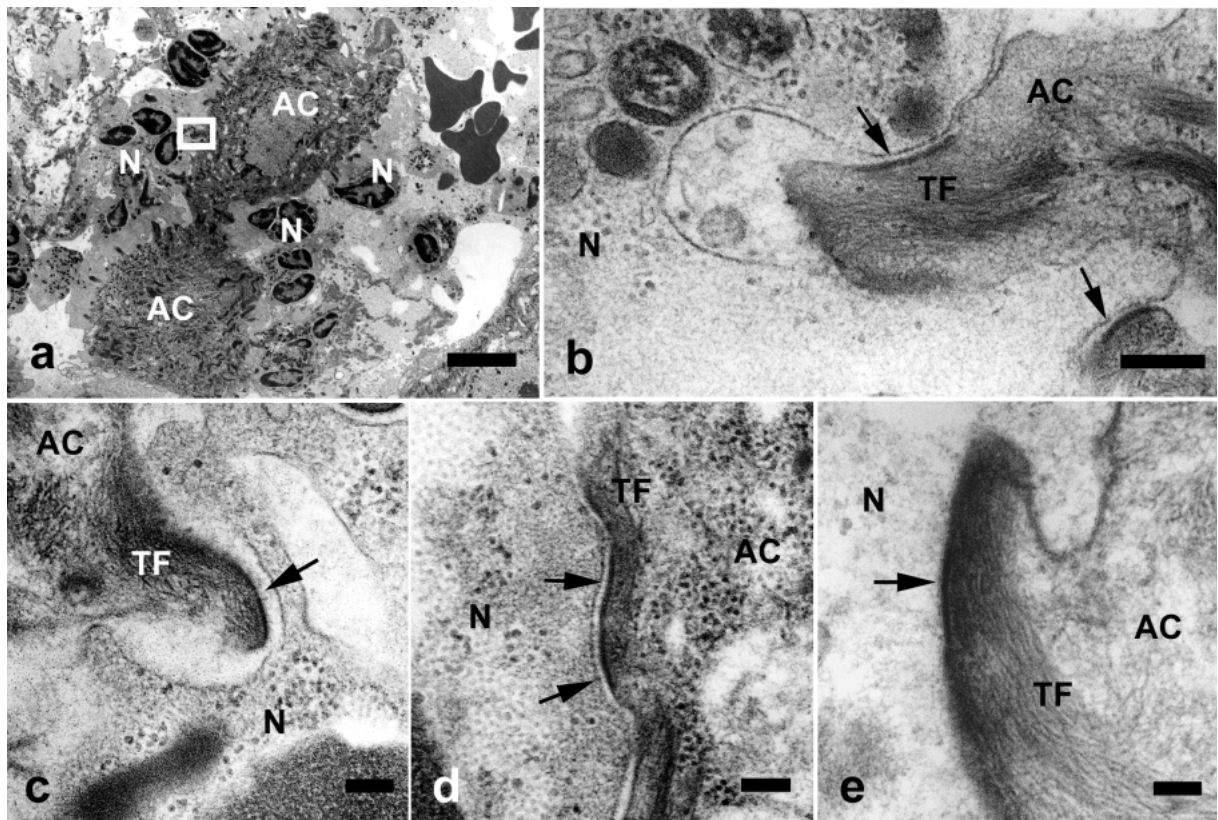


Fig. 2. Electron micrograph of an epidermal lesion of dogs#1-4. In dog#1, two acantholytic cells (AC) are observed and surrounded by several neutrophils (N). Bar=5  $\mu$ m (Fig. 2a). Close up view of the rectangle in Fig. 2a shows half-desmosome structures (arrows) on the surface of an acantholytic cell (AC) in the invagination of plasma membrane of a neutrophil (N) (Fig. 2b). Tonofilaments (TF) are inserted into the attachment plaque of the half-desmosomes. Bar=200 nm. In dog#2, the half-desmosome on the surface of an acantholytic cell is localized in the invagination of plasma membrane of neutrophils (N). Bar=100 nm (Fig. 2c). Dogs#3 and #4, respectively show the same feature as Dog#1 and 2 (Fig. 2d and e). Neutrophils (N) seem to closely contact to the half-desmosomes on the surface of acantholytic cells (AC). Bar= 200 nm. Uranyl acetate and lead citrate counterstaining.

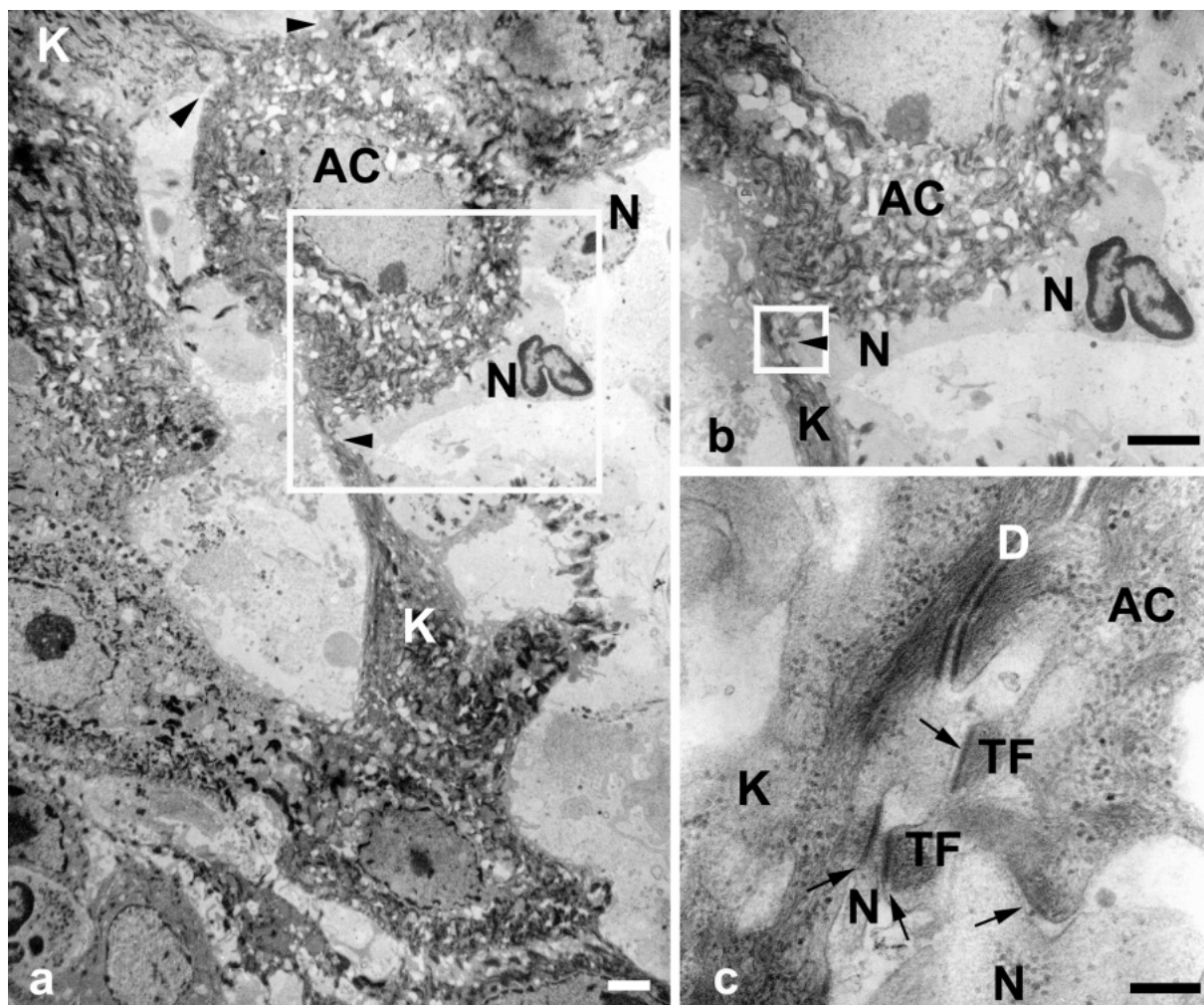


Fig. 3. Electron micrograph of an epidermal lesion showing the early phase of acantholysis of dog#1. An acantholytic cell (AC) is partially attached to the bottom of the intraepidermal cleft. Arrowheads indicate the contact point between AC and keratinocytes (K). Neutrophils (N) surround the acantholytic cell. Bar=2  $\mu$ m (Fig. 3a). Close up view of the rectangle in Fig. 3a shows a neutrophil (N) extending its cytoplasm toward the cell-cell contact site (arrowhead) between the acantholytic cell (AC) and adjacent keratinocyte (K) (Fig. 3b). Bar=2  $\mu$ m. Close up view of the rectangle in Fig. 3b shows a neutrophil elongates its pseudopodia between an acantholytic cell (AC) and an adjacent keratinocyte (K). The half-desmosomes (arrows) have intact attachment plaque with attached tonofilaments (TF) (Fig. 3c). An intact desmosome (D) is also seen in the upper side of this photograph. Bar =200 nm. Uranyl acetate and lead citrate counterstaining.

inocytes and seemed to split desmosomal cell-cell adhesion (Fig. 3c). Elongation of neutrophil cytoplasm toward the intercellular gap between keratinocytes was also observed in an epidermal lesion in a case No. 4, and the plasma membrane of the neutrophil contacted with a half-desmosome of a keratinocyte (data not shown).

Furthermore, we wanted to examine the roles of neutrophils during mid-to-late phases of acantholysis in canine PF. We therefore analyzed the cell surface of free-floated acantholytic keratinocytes, which were surrounded by multiple neutrophils in a microscopic pustule from a case No. 1 (Fig. 4a). We found that neutrophil granules were released to the intercellular spaces between the neutrophil and acantholytic keratinocyte (Fig. 4b). In these intercellular spaces, several

half-desmosome structures lost their smoothness in appearance and seemed to be degenerated by granular enzymes. The electron microscopic findings were summarized in Table 1.

## DISCUSSION

Canine PF has been histopathologically referred to as a pustular skin disease with intraepidermal separation of keratinocytes and infiltration of granulocytes since the 1970s [4, 7, 11]. Although a previous paper described that circulating IgG in three dogs with PF caused intraepidermal blisters similar to that seen in PF [12], the roles of infiltrated granulocytes in cutaneous lesions in canine PF have not been

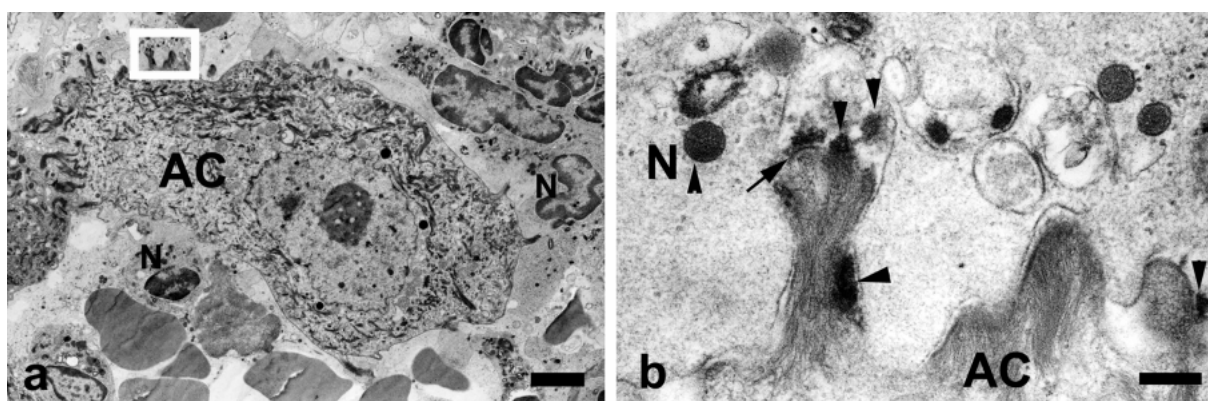


Fig. 4. Electron micrograph of an epidermal lesion showing the mid-to-late phases of acantholysis in dog#1. Multiple neutrophils (N) attach to a freely-floated acantholytic cell (AC). Bar=2  $\mu$ m (Fig. 4a). Close up view of the rectangle in Fig. 4a shows neutrophilic granules (arrowheads) in the intercellular spaces between neutrophil (N) and half-desmosome structures (arrow) (Fig. 4b). These half-desmosomes lost their smoothness in appearance. Bar=200 nm (Fig. 4b). Uranyl acetate and lead citrate counterstaining.

cleared. In this study, we found that all half-desmosomes on the surface of acantholytic cells were contacted with plasma membrane of neutrophils. Moreover, in the early phase of acantholysis, neutrophil inserted its pseudopodia into the intercellular gap between adjacent keratinocytes. These findings suggested that neutrophils preferentially attached to plasma membrane of keratinocytes including desmosomes, and may play a part in dissociation of keratinocytes. The interaction between acantholytic cells and neutrophils suggested if neutrophils have an auxiliary role in dissociation of keratinocytes besides antigen-autoantibody interaction in canine PF. On the other hand, in the mid-to-late phases of acantholysis, cytoplasmic granules of neutrophils were recognized in the intercellular spaces between the neutrophil and acantholytic keratinocyte. Thus, it is also intriguing if neutrophilic granules secreted towards half-desmosomes could contribute to the clearance of the desmosomal structures resulting in the defects of adhesive properties of keratinocytes in canine PF.

Our findings also indicated that half-desmosome structures, which had intact attachment plaques and attached tonofilaments, were recognized on cell surface of the acantholytic keratinocytes contacted with neutrophils. The half-desmosomes without retraction of tonofilaments have also been recognized on the apical surface of acantholytic splits in human patients with pemphigus vulgaris (PV), another subtype of pemphigus, and a mouse model of PV [5, 8, 14]. As IgG autoantibodies bind the extracellular region of Dsg3 [14, 15], it has been thought that IgG binding causes desmosomal split by direct inhibition of desmosomal cell-cell adhesion, thereby leading to blister formation in the PV. Conversely, several previous papers have described that serum IgG in human patients with PV and PF may trigger intracellular events and cause tonofilament retraction from the attachment plaque and subsequent internalization of desmosomes *in vitro* in cultured keratinocytes [1–3, 18]. In previous EM study of human PF, it was described that the tonofilaments were frequently separated from attachment

plaque of desmosomes in acantholytic lesions in the Malpighian layer [19]. Since all of the more than twenty half-desmosomes had intracytoplasmic dense plaques, and retraction of tonofilaments was unremarkable in present study, it is suggested that intercellular signaling triggered by IgG autoantibodies is not essential in disruption of desmosomal cell-cell adhesion in canine PF.

In summary, our ultrastructural findings suggested that neutrophils preferentially contacted to desmosomal structures and seemed to play a part in separation of adjacent keratinocytes and degeneration of split-desmosomes in cutaneous lesions in canine PF. To the best of our knowledge, this is the first report to provide findings which lead speculations of the functional roles of neutrophils in the development of cutaneous lesions in canine PF. Future studies are expected to define the relationship between autoantibodies and neutrophils as well as the mechanisms for triggering the neutrophil infiltration in pustular lesions in canine PF.

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