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NOTE

Internal Medicine

Detection of granzyme B in CD3-positive cells infiltrated in lesional skin of a dog with erythema multiforme associated with zonisamide

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ABSTRACT. In human erythema multiforme (EM), cytotoxic T lymphocytes (CTLs) play an essential role in the pathogenesis. In canine EM, immunohistochemical staining with anti-CD8 antibody using frozen sections has shown the involvement of CTLs; however, CTL infiltration has never been quantitatively analyzed. We herein quantitatively analyzed CTL infiltration by immunohistochemical staining with granzyme B and CD3 antibodies using paraffin sections of a dog with EM associated with zonisamide. The present results indicated approximately 70% of cells at the border between the epidermis and dermis consisted of CTLs. Detection of granzyme B and CD3 using paraffin sections employed in this study can be a clinically applicable method for detecting CTLs.

KEY WORDS: CD3, cytotoxic T lymphocytes, dog, erythema multiform, granzyme B

Erythema multiforme (EM) is characterized by the acute onset of a cutaneous inflammatory reaction that is histopathologically characterized by epidermal apoptosis and lymphocytic satellitosis [12]. Various drugs, including zonisamide, have been identified as causative agents of EM in dogs [1, 10]. In humans, EM was mediated by cytotoxic lymphocyte responses against keratinocytes [7, 14]. In dogs with EM, the infiltration of CD3 or CD8-positive lymphocytes in the lesion has been shown by immunohistochemical staining [2], however, cytotoxic T lymphocyte (CTL) infiltration has never been quantified. Although CD8 is an essential marker for CTLs, immunohistochemical staining requires frozen sections, which prompts us to develop alternative methods with paraffin sections. In the present report, we quantified CTL infiltration in a dog with EM associated with zonisamide by immunohistochemical staining of granzyme B and CD3 with paraffin sections.

An eight-year-old, castrated, 12.5 kg, male mixed-breed dog was presented with a four-month history of progressive dermatitis with erosion. At presentation, the dog had multifocal erythema with erosions, ulcers and crusts on the abdomen and groin (Fig. 1a). Focal erosion with a crust was also observed on the right medial axilla. The dog was receiving three different drugs for immune-mediated encephalitis: zonisamide (Consave®; DS Pharma Animal Health, Osaka, Japan) 4.2 mg/kg 12 hr, prednisolone (unknown generic name) 0.2 mg/kg 24 hr, and famotidine (unknown generic name) 0.8 mg/kg 12 hr for eight months.

A cytological evaluation of skin lesions revealed a number of denatured/non-denatured neutrophils with a few macrophages. A complete blood count showed a high platelet count (709,000/µl, reference range 200,000−400,000/µl). Serum chemistry showed an elevated alkaline phosphatase level (408 U/l, reference range 49−298 U/l), which was likely due to the administration of prednisolone. A histopathological examination of skin biopsy specimens revealed cytotoxic interface dermatitis with epidermal keratinocyte cell death and lymphocytic satellitosis (Fig. 2). Since clinical signs and histopathology supported a diagnosis of EM, zonisamide was suspected as a causative agent, which was consequently withdrawn and replaced with levetiracetam (E Keppra®; UCB Japan; Tokyo, Japan) 20 mg/kg 12 hr. Prednisolone and famotidine were continued. Skin lesions improved within a few days and reached complete remission within 2 weeks (Fig. 1b).

Paraffin-embedded skin sections were stained immunohistochemically with anti-human cleaved caspase-3 (1:200, 5A1E; Cell Signaling Technology, Danvers, MA, USA), anti-human CD20 (1:1,000; Thermo Fisher Scientific, Waltham, MA, USA), anti-human granzyme B (1:400; Spring Bioscience, Pleasanton, CA, USA) and anti-human CD3 (1:200, clone CD3-12; Abcam,

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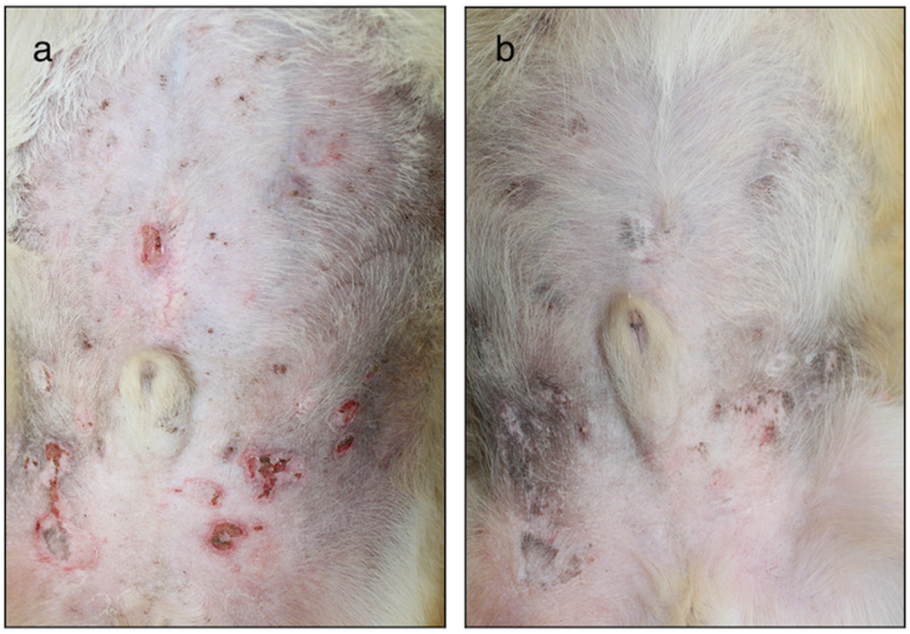

Fig. 1. Skin lesions on the abdomen and groin. **(a)** Erosions, ulcers and crusts on the abdomen and groin on the day of presentation. **(b)** The healing of erosive lesions two weeks after the discontinuation of zonisamide.

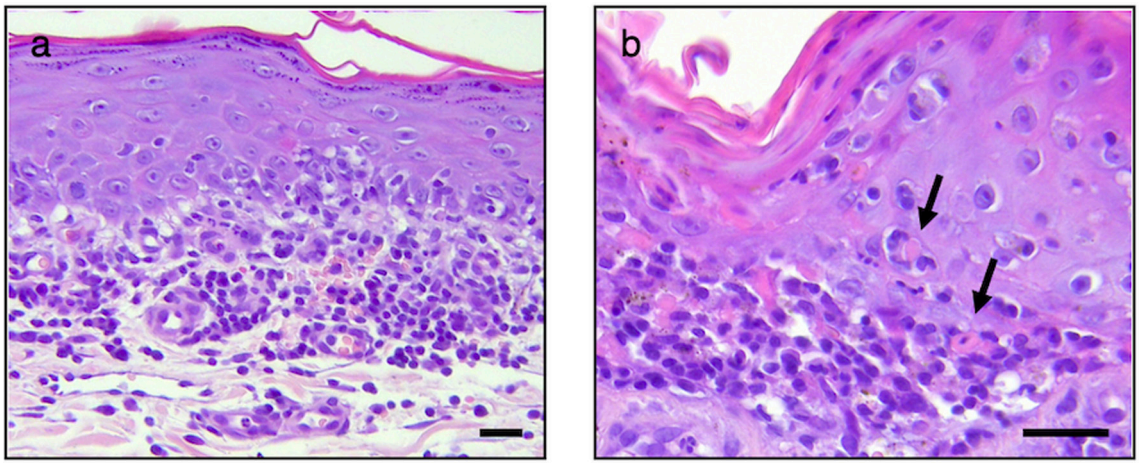

Fig. 2. Photomicrographs of lesional skin sections stained with hematoxylin and eosin. **(a)** Dermal infiltration of lymphocytes in cytotoxic interface dermatitis. **(b)** Epidermal keratinocyte cell death and lymphocytic satellitosis (arrows). Scale bar=50 µm.

Cambridge, UK) antibodies for a more detailed investigation. An immunoenzyme method was employed for cleaved caspase-3 and CD20. In addition, granzyme B and CD3 were detected by an immunofluorescence method using secondary antibody-conjugated Alexa Fluor 594 goat anti-rabbit IgG and fluorescein isothiocyanate goat anti-rat IgG antibodies. In immunohistochemical staining, non-specific reactions were blocked with 10% normal goat serum in PBS. The antibodies used in the present study were all confirmed to cross-react with canine counterparts in other studies [5, 6, 8, 13].

Immunohistochemistry showed the presence of cleaved caspase-3 in a necrotic keratinocyte (Fig. 3). There were no CD20-positive cells among mononuclear cells in the dermis (data not shown). Furthermore, fluorescent double-immunostaining revealed that infiltration of granzyme B and CD3 double-positive cells in the skin lesions (Fig. 4). Approximately 70% of cells infiltrating at the border between the epidermis and dermis were positive for both granzyme B and CD3. Besides, about 20% of cells were granzyme B-positive/CD3-negative and 10% of cells were granzyme B-negative/CD3-postitive.

Although the present case was not challenged with zonisamide as additional exposure might result in a more severe reaction, we diagnosed the present case with EM secondary to zonisamide administration based on a clear improvement in skin lesions by discontinuing zonisamide. In human EM, keratinocyte cytotoxicity was induced by soluble mediators, such as Fas ligand, granzymes, perforin, and granulysin, from CTLs and natural killer (NK) cells [7, 9, 14]. In the skin lesions of dogs with EM, the

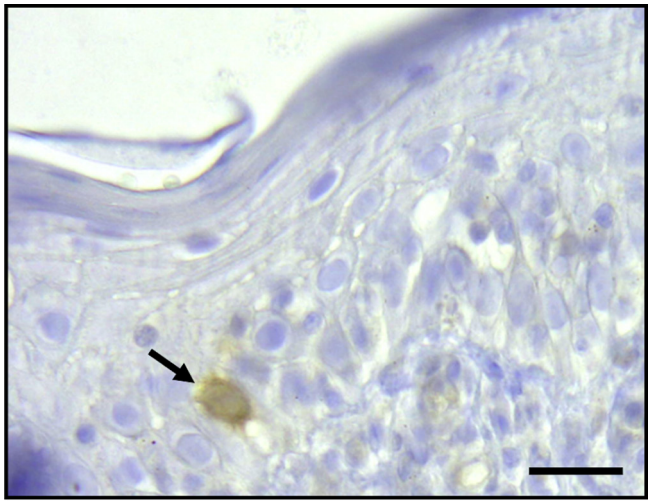

Fig. 3. A photomicrograph of a lesional skin section stained by an anti-cleaved caspase-3 antibody. A necrotic epidermal keratinocyte was positive for cleaved caspase-3 (arrow). Scale bar=50 µm.

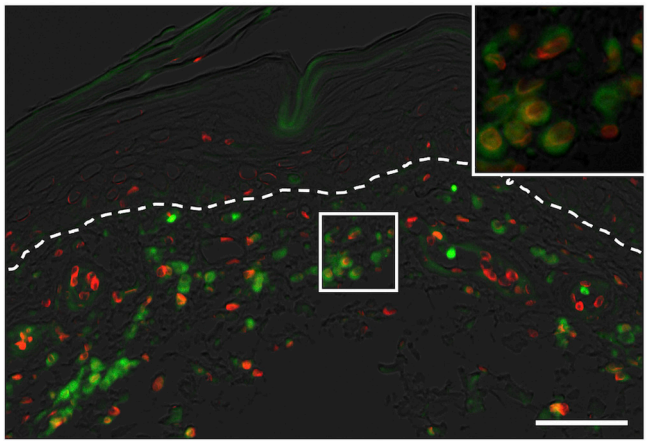

Fig. 4. A photomicrograph of a lesional skin section double-stained by fluorescent immunostaining with granzyme B/CD3. Double staining with granzyme B (red) and CD3 (green) immunofluorescence. The majority of cells infiltrating the border between the epidermis and dermis were positive for both granzyme B and CD3 (inset; 2× magnified image). Dotted lines are show the basal layer of epidermis. Scale bar=50 µm.

keratinocyte apoptosis with the infiltration of lymphocytic cells have been demonstrated [4]. A previous canine study showed the infiltration of CD3 or CD8-positive lymphocytes in canine EM; however, images of the immunohistochemical staining were not provided, so the degree of CTL infiltration was not clear [2]. Thus, prospective studies should focus on quantitative analysis with a more clinical detection method using paraffin but not frozen sections. For this purpose, we performed immunohistochemical double-staining with granzyme B and CD3 to identify CTLs [3, 11]. The present results revealed that approximately 70% of cells at the border between the epidermis and dermis consisted of granzyme B and CD3 double-positive cells, suggesting that CTLs mediate the cytotoxicity against target keratinocytes via granzyme B. Immunohistochemical analysis indicated the presence of granzyme B-positive/CD3-negative cells in the skin lesions. In human EM, not only CTLs, but also NK cells were identified in the lesional skin [7, 9], therefore, granzyme B-positive/CD3-negative cells found in the present case might be NK cells. Further studies on the involvement of NK cells and other apoptosis-related factors, such as perforin and granulysin, produced by CTLs and NK cells will provide a more detailed understanding of the pathophysiology of canine EM.

In summary, this case report is the first to show the degree of CTL infiltration by detecting granzyme B and CD3 in the paraffin sections from a dog with EM associated with zonisamide. The immunohistochemical method performed herein will be practically helpful to provide insights into the immunopathogenesis of various skin diseases mediated by CTLs.

CONFLICTS OF INTEREST. The authors declare that they have no conflicts of interest related to the subject materials discussed in this article.

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