Cells of the skin immune system in dogs with atopy

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Abstract

Twenty-five dogs with signs of atopic dermatitis were included in this study. Additionally, 10 healthy dogs were chosen as healthy skin controls. Skin biopsy specimens were taken from these dogs and evaluated for the following cells: basal cell layer including the number of mitotic figures in this layer, spinous cell layer, macrophages, melanocytes, mast cells and dendritic cells. Identification of mast cells and dendritic cells was performed by means of immunohistochemistry. Histological and statistical investigations showed that the number of mitotic figures in the basal cell layer as well as the number of mast cells, melanocytes, dendritic cells and macrophages was significantly higher in the skin of dogs with atopic dermatitis compared to the healthy dogs ($p < 0.01$). This finding indicates multilateral quantitative activation within the cellular elements of the skin immune system. Furthermore, marked morphological heterogeneity and distinct degranulation patterns observed among mast cells in atopic skin points to their significant functional activation and confirms that canine atopic dermatitis is still notably a mast cell-dependent disease.

Canine, SIS, atopic dermatitis, mast cells, epidermal dendritic cells

In the past 10 years, it has been observed that allergic diseases are becoming increasingly frequent both in animals and human beings. Atopic dermatitis is one of the most common canine allergic skin diseases and one of the most difficult to diagnose and treat. It has been estimated that up to 10-15% of the canine population and up to 15% of people are affected with atopy (Nagle et al. 2001). It seems to be a problem of civilization connected with rising environmental pollution. The fact that our pets spend much more time indoors exposed to dust mite antigens is also a possible reason for the increased prevalence of atopic dermatitis (Pedersen 1999). In the field of human medicine, the 'hygiene hypothesis' and 'western' life style contribute to the allergic immunological profile (Th2 lymphocytes domination) (Lasek 2005; Roosje 2005). Despite extensive research and experiments both in human and veterinary medicine, the exact pathogenesis of atopy is still unclear.

The skin is the largest organ of the body and functions as an important immunological unit at the same time. The skin immune system (SIS) is a microenvironment of many different processes taking place both under physiological and pathological conditions (Bos 1997; Scott et al. 2001). It is well understood that the phenomenon of atopy as immunodermatosis should not be analyzed independently of the skin immune system (Bos 1997; Hill and Olivry 2001). Mast cells as a very important cellular element of the skin immune system have been intensively studied in the context of allergic diseases including atopy for years. Their main biological role has been commonly connected with anaphylactic reactions. Currently, mast cells as well as T lymphocytes are considered to be major effector cells in the course of atopy, being responsible for the immune cell infiltration into atopic skin (Hill and Olivry 2001). Despite a great deal of scientific research

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concerning the pathogenesis and the course of atopy, this complicated process includes many areas where our knowledge is not yet complete. Moreover, the histopathology of the skin lesions of canine atopic dermatitis is considered nonspecific. The purpose of this study was to examine the immune cell system in canine atopic dermatitis on the basis of the occurrence of mast cells, dendritic cells, macrophages and other cells of the SIS in the skin of dogs with atopic dermatitis. Estimating the numbers of immune cells and their morphology seems to be a reliable model for describing the structure and the function of the cellular elements of the skin immune system in allergic and inflammatory skin diseases such as atopic dermatitis. In the course of this study particular attention was devoted to mast cells. They were investigated both: quantitatively, in the context of other cellular SIS elements and at the ultrastructural level in order to describe their degranulation activity and degranulation patterns in the skin of dogs with atopic dermatitis.

Materials and Methods

Animals

25 dogs of different breeds and both sexes (1 Yorkshire Terrier – male, 2 Dachshunds– male and female, 7 Labrador Retrievers – three males and four females, 4 Golden Retriever - females, 1 Akita - male, 4 West Highland White Terriers - three males and one female, 1 Boxer - female, 4 German Shepherd - males, 1 American Staffordshire Terrier - male), ages (1.5 and 5-6 years) with atopic dermatitis were included to this study. Additionally, 10 healthy, 1-5 years old dogs of different breeds (1Yorkshire Terrier - male, 3 Labrador Retriever - males, 2 Golden Retriever - male and female, 2 West Highland White Terrier - females, 1 German Shepherd - male, 1 mixed breed - male), were chosen as control group. This study was carried out at a private animal practice in Poland.

Prior to the study, all dogs underwent a thorough physical examination particularly focused on dermatology.

Dogs were diagnosed with atopic dermatitis, when the Willemse and Prélaud clinical criteria of this disease were met. More than 3 major and at least 3 minor Willemse criteria and at least three Prélaud criteria were met in case of all dogs with atopic dermatitis. Similar pruritic diseases (e. g. adverse food reactions, insect bite hypersensitivities, and scabies) were ruled out by standard diagnostic and therapeutic methods (8 week-long elimination diet, skin scrapings, acaricidal therapy, flea control). The selected dogs had pruritus with no lesions, some had erythema and papules. Secondary skin lesions such as excoriations, alopecia, lichenification and hyperpigmentation were less frequent. Dogs with strong secondary infection of the skin were excluded from the study. If primary or secondary skin lesions were present, cytological examination was performed.

Biopsy specimens were taken using local anaesthesia (lidokaine injectable 2%) from various nonlesional parts of the body such as the ventral abdomen, the axillary region, the inguinal region according to the judgment of the examiner. The ventral abdomen region was preferred because of the technical easiness of sample collection and because the fact of obtaining healthy dogs skin biopsies from similar, sparsely haired, ventral abdomen area. The skin was biopsied using a 6-mm punch biopsy instrument or a scalpel blade. Each specimen was cut into 3 pieces immediately after sampling.

Haematoxyline and eosin staining

One part of the specimen was fixed in 10% buffered formalin, paraffin-embedded, sectioned with a Zeiss Microm HM 340E to 5 μm sections, stained with haematoxyline and eosin (Shandon) and analyzed using light microscope (Axio Imager A1).

Immunohistochemistry

One part of the paraffin sections was dewaxed and rehydrated. Monoclonal antibodies against mast cell tryptase were employed, using immunoperoxidase cell staining. Heat-induced antigen retrieval was performed: slides were incubated in Target retrieval solution pH = 9. 0 (Dako, Denmark) for 20 min. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide for 5 min. Then the slides were washed with TBS (Tris buffer solution) for 5 min each, incubated with the primary antibody for 1 h at room temperature and washed with three changes of TBS for 5 min. Detection was performed with EnVision™ Systems (Dako, Denmark). Then, sections were counterstained with Mayer's haematoxyline and observed by means of light microscopy (Axio Imager A1).

The second part of skin biopsy sample was placed into cryostat (Leica CM1850UV) and cut up into 5 μm sections. Sections were air dried and fixed in hydrogen peroxide to quench endogenous peroxidase. Nonspecific binding was blocked by preincubation for 10 min with blocking serum (Santa Cruz Biotechnology, USA). The antibodies to canine cluster of differentiation 1a (CD1a) and canine major histocompatibility complex class II (MHC II) provided by LABL (Dr. Peter F. Moore) were applied and sections were incubated for 30 min at room temperature. Then slides were washed with 3 changes of TBS for 5 min each. Detection was performed with EnVision™ Systems (Dako, Denmark). Then the sections were lightly counterstained with Mayer's haematoxyline and observed by light microscopy (Axio Imager A1).

Ultrastructural analysis

The third part of each specimen was fixed in 2.5% glutaraldehyde in phosphate buffer (ph 7. 4). After 2 h of post fixation in 2% osmium tetroxide, routine dehydratation in acetone series was performed. Fixed tissues were embedded in epoxy resin (Epon 812) for the transmission electron microscopy examination (Tesla BS500). Ultrathin 70 nm section were contrasted with uranyl acetate and lead citrate and viewed in electron microscope.

Statistical analysis and cell quantification

Biopsy specimens stained with H&E were evaluated for number of the following cells: basal cells including the number of mitotic figures in this layer, spinous cell layer, melanocytes and macrophages. Biopsy specimens immunohistochemically stained for mast cell tryptaze were analyzed for the number and topography of the mast cell population. Specimens exhibiting the expression of canine CD1a and canine MHCII were examined with respect to the number and localization of dendritic cells. Histometric analysis covered 10 high-powered fields -HPF (× 400) from each slide including both epidermal and dermal cell types. Each sample is reported as the mean value of the counted cells in 10 HPF by means of morphmetric programme (AxioVision 4 Module AutoMeasure) . Mann-Whitney test (MWT) was used to compare cell numbers in healthy dogs and dogs with atopic dermatitis ($p < 0.01$). Spearman ranking correlation between the examined cell populations was done in dogs with atopic dermatitis ($p < 0.05$).

Results

Histological and statistical investigations showed that the cell counts were significantly different in the skin of dogs with atopic dermatitis compared to the control healthy dogs (Table 1). In the epidermis of the dogs with atopic dermatitis there were more mitotic figures in the basal cell layer ($p < 0.01$), increased number of spinous layer cells and the number of epidermal dendritic cells and melanocytes ($p < 0.01$). In the area of dermis there was a rise in the number of mast cells and macrophages ($p < 0. 01$).

Table 1. Statistics of cell populations in healthy dogs and dogs with atopic dermatitis.

		Mean	SD	SE	95% CI	Min	Max	*P* value
BC	H	18.46	1.37	0.61	16.75–20.17	14	24	0.0452
	A	20.99	2.50	0.52	19.91–22.07	15	28	
BCM	H	6.58	0.53	0.24	5.93–7.23	3	9	0.00002
	A	9.52	1.21	0.25	8.99–10.04	6	14	
SS	H	11.26	2.80	1.25	7.78–14.74	5	18	0.00002
	A	18.54	2.13	0.44	17.62–19.46	13	24	
EDC	H	1.90	0.23	0.10	1.61–2.19	1	4	0.00002
	A	10.27	1.25	0.26	9.73–10.81	6	16	
M	H	4.36	0.73	0.33	3.45–5.27	2	7	0.00002
	A	9.73	1.13	0.24	9.24–10.22	6	13	
MC	H	2.38	0.28	0.12	2.04–2.72	1	4	0.00002
	A	9.29	1.16	0.24	8.79–9.79	6	12	
MF	H	4.06	0.21	0.09	3.80–4.32	2	6	0.00002
	A	7.58	0.63	0.13	7.31–7.85	4	11	
FB	H	11.48	0.29	0.13	11.11–11.85	8	14	NS
	A	11.06	0.91	0.19	10.66–11.45	6	14	
FC	H	16.12	1.87	0.84	13.79–18.45	10	22	0.00002
	A	11.43	1.37	0.29	10.84–12.03	6	18	

SD - standard deviation; SE - standard error, 95% CI - 95% confidence intervals of the means of the cells; Min -minimum value; Max - maximum value; P value - value obtained for the Mann-Whitney test; BC - number of stratum basale cells; BCM - number of mitotic figures within stratum basale; SS - number of stratum spinosum cells; EDC - epidermal dendritic cells; M - melanocytes; MC - mast cells; MF - macrophages; FB - fibroblasts; FC - fibrocytes; H - healthy dogs; A - dogs with atopic dermatitic, NS - not significant.

Histopathological examination of the skin biopsy specimens indicated obvious activation of the cells in the germinal layers of the epidermis. The intensification of the keratinisation process was also demonstrated as the stratum corneum was wider compared to healthy control and included delaminations (Plate I, Fig. 1).

Moreover, significant correlation between the count of stratum spinosum cells and melanocytes ($p < 0.05$) was observed in atopic dogs. The second examined nonepithelial group of cells within the germinal layers of the epidermis much more numerous in the skin with atopy compared to the healthy control, was the epidermal dendritic cell population (EDC) ($p < 0.01$). EDC were scattered in the skin sections of the atopic dogs throughout the suprabasilar layers of the epidermis and sometimes they were gathered in clusters.

Furthermore, in the perivascular area of the skin with atopic dermatitis mixed inflammatory cell infiltrate was observed, consisting mainly of mast cells, dendritic cells, macrophages and lymphocytes. Dermal dendritic cells were present throughout the dermis (most frequently in a perivascular location) in the close proximity to mast cells and macrophages.

In the specimens collected from the atopic dogs, dermal mast cell counts were significantly higher than in the control healthy dogs. In the subepidermal and perivascular compartment of the skin, mast cells were seen as individual cells but more often as cell clusters. The immunoperoxidase staining with monoclonal antibodies resulted in intense staining of mast cell granules, which provided precise localization of the mast cell in the skin but also the information about topography of granules within the individual cells. Secretory granules were either distributed at the margins of the cells or filled densely the whole cell obscuring the cell nucleus. In close proximity to some described mast cells free granules were found in their cell exterior which is characteristic for anaphylactic degranulation (Plate I, Fig. 2). Electron microscopic studies revealed that mast cells from canine skin with atopic dermatitis exhibited piecemeal (PMD), anaphylactic and mixed pattern of granules release.

Granules piecemeal losses were focal from single granules or had features of complete losses of granule contents from variable numbers of secretory granules (Plate II, Fig. 3). One can appreciate typical PMD, enlarged, non-fused, empty and partially empty granules. Mast cells undergoing anaphylactic degranulation were characterized by visible granule to granule and granule to cytoplasmic membrane fusions as well as granules present near the given mast cell in the extracellular milieu.

The number of macrophages in the skin of atopic dogs was higher than in healthy dogs and they were situated both in a perivascular location and just under the epidermis. The macrophages were found near the mast cells in the perivascular location.

Electron microscopy showed that macrophages from the dogs with atopic dermatitis had abundant lyzosomes and their outer membrane was highly pleomorphic and exhibited numerous structures reminiscent of microvilli. These findings reflect intense cell activity.

Discussion

Atopy, atopic state (according to The American College of Veterinary Dermatology) is a genetically-predisposed tendency to develop IgE-mediated inflammatory and pruritic allergic skin disease with characteristic clinical features (Gueck et al. 2004). It is believed to be a type I hypersensivity reaction with a prominent late phase reaction (LPR) (Olivry et al. 1997; Roosje et al. 2004).

Atopy, as an allergic skin disease, should be considered in the context of the skin immune system (SIS) (Bos 1997). According to recent studies (Scott et al. 2001; Gross et al. 2005, canine atopic dermatitis (AD) skin biopsies most often exhibit an inflammatory pattern characterized as mixed perivascular dermatitis which was seen also in our study. Moreover the present study confirmed the multilateral activation within the cells of the skin immune system, as far as their numbers and cell activation states are concerned. This confirms an active immunological response to invading allergens within the skin in the course of atopy. In the area of the atopic dogs' epidermis, an intensive mitotic activity among cells of the stratum basale and high number of the stratum spinosum cells supports the theory of the importance of the keratinocytes as auxiliary cells which influence the immunological

reaction and collaborate with other SIS immunocompetent cells (Reedy et al 1997; Scott at al. 2001). They do not function only as a mechanical barrier but are a primary source of interleukin-1 and other cytokines as well as growth factors (Lasek 2005). This activation within the atopic epidermis might also be indirect proof of transepidermal contact with the antigen during atopy. Invading allergens activate keratinocytes and epidermal dendritic cells, their main partners in the initiation of the immune response within the epidermis (Olivry and Hill 2001 Marsella et al 2006; Marsella 2006). On the other hand, both the increased mitotic activity and the observed hyperkeratinization process within atopic epidermis should be also treated as just an adaptation mechanism. It implies an altered epidermopoesis of inflammatory origin connected with the chronicity of the process and characteristic not only for atopy but also for other inflammatory, long lasting diseases of the skin (Scott at al 2001).

The germinative compartment of the epidermis is a perfect field for the existence of nonepithelial cells such as melanocytes and Langerhans cells. Much more numerous melanocytes in the skin of atopic dogs compared with the healthy dogs and a significant positive correlation between their number and the count of the stratum spinosum cells may be a result of growth factors and cytokines production by activated keratinocytes (IFNγ, Il-8, MSH) (Sulaimon and Kitchell 2003). Postinflammatory hyperpigmentation during atopic dermatitis is a common consequence of an inflammatory insult.

Another group of cells whose quantity was much higher in the group of atopic dogs were the dendritic cells, cells that appear to be most important besides the Th2-lymphocytes and mast cells in the pathogenesis of canine AD. The population of the epidermal dendritic cells (EDC) present in the epidermis interacts with the keratinocytes and lymphocytes during the initial phases of the immunological response (Katou et al. 2000). They act as sentinels of the skin immune system and they are major antigen presenting cells especially after an epicutaneous antigen challenge (Cutler et al. 2001; Lipscomb et al. 2002; Day 2006). The finding that the epidermis of atopic dogs contains significantly more Langerhans cells than that of the control healthy dogs strongly suggests a transepidermal way of contact (Marsella 2005). The increased number of the dendritic cells both in the epidermis and dermis confirms that the process of allergens' presentation during atopy is taking place not only in lymph nodes but also *in situ* in the skin. It is characteristic not only for atopy but also for a considerable group of inflammatory dermatoses with a prominent cell-mediated immune response (Olivry et al. 1996; DeBoer 2004).

During skin inflammation, the dermal perivascular region is the area of the most significant and dynamic changes. Some authors even detail elements connected with postcapillary venules in a subsystem named the dermal microvascular unit (DMU). DMU consists of postcapilar venules, endothelial cells, perivascular mast cells, macrophages and dermal dendritic cells. It is considered as the centre of immunological reactivity in most immunodermatoses including atopy (Bos 1997).

The most prominent changes within the dermis of atopic dogs were connected with the mast cell population. IgE-mediated mast cell release of performed mediators (e.g. histamine) results in immediate type hypersensitivity reactions and evoke the late phase reaction (LPR), which may be of much greater importance for atopic dermatitis (Marsella and Niclin 2001). The LPR occurs within hours after an antigen challenge and is caused by mast cell release of chemotactic mediators (leukotrienes, Il-4, TNFα) leading to dermal infiltration of inflammatory cells (Olivry et al. 1997; Hill and Moore 2000; Hill et al. 2001; Olivry et al. 2001). Ultrastructurally anaphylactic, piecemeal and mixed degranulation patterns were distinguished among mast cells in atopic skin in this study. There was marked domination of 'piece meal' and 'mixed' type of degranulation in the atopic skin. This observation is consistent with other studies that provide evidence that during atopic dermatitis as a chronic inflammatory skin disease, the predominance of

the piece meal over anaphylactic degranulation occurs (Dvorak 2005). Those findings indicate that in sites of IgE-mediated reactions within the skin, mast cells undergo typical changes like rapid anaphylactic degranulation but also slower alternation of their secretory granules (Kaminer et al. 1995). This could be explained by the presence of not only IgE antibodies, but also many other inflammatory mediators such as complement components, cytokines and neuropeptydes within the atopic skin that can provoke PMD (Kaminer et al. 1995; Dvorak 2005; Nuttall 2006).

Relatively new findings have been reported underlying the crucial role of components of atopic inflammatory infiltrate other than mast cells, predominately Langerhans cells and lymphocytes Th (Nuttall 2006). However, as indicated, marked quantitative activation and morphological heterogeneity within the mast cell population in atopic skin supports the theory that mast cells should be considered as the main effector cells of the atopic state contributing to a prominent mixed inflammatory cell infiltrate (Hammerberg et al. 2001; Nuttall 2006).

The macrophage counts are higher in atopic skin than in healthy controls suggesting they also possibly play an important role in atopic dermatitis particularly in chronic phase due to their contribution to Th1-lymphocytes domination (Olivry et al. 1997; Day 2006).

Complete summary of the skin immune response in the course of atopic dermatitis is not easy. The reason for that is the fact that during the pathogenesis of atopy, both cellular and humoral interplays are interwoven and interdependent and they often take place at the same time. Nevertheless, atopy gives a perfect background for analyzing the cellular elements of SIS. Taking into consideration the striking ultrastructural heterogeneity within the mast cell population as well as their distinct morphological degranulation patterns, it is apparent that mast cells remain key regulatory elements in the atopic inflammatory cascade. The mechanism by which they influence other cellular elements of SIS should become the subject of further investigation.

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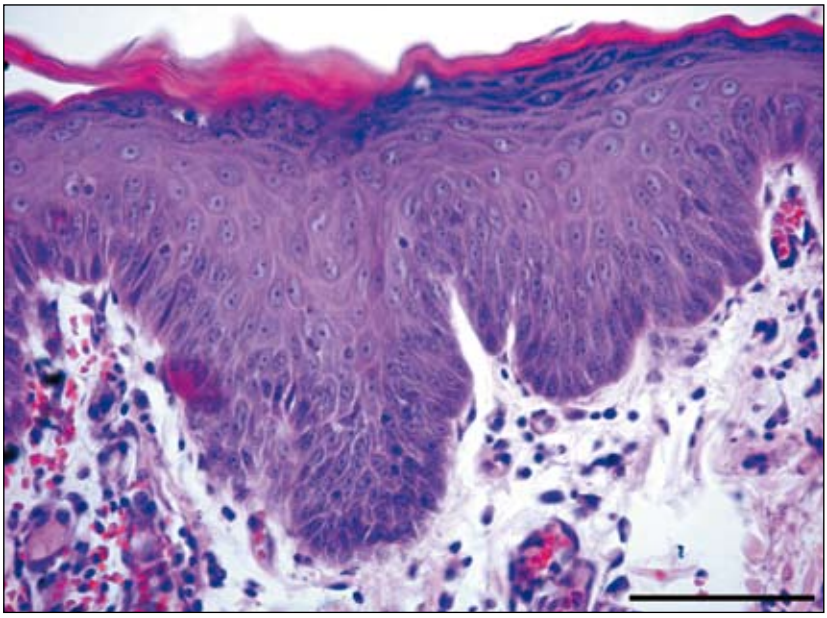

Fig. 1. Canine skin with atopic dermatitis. There is epidermal thickening hyperkeratosis and mixed inflammatory infiltrate in the subepidermal and perivascular compartment of the skin (H&E staining, magnification × 400).

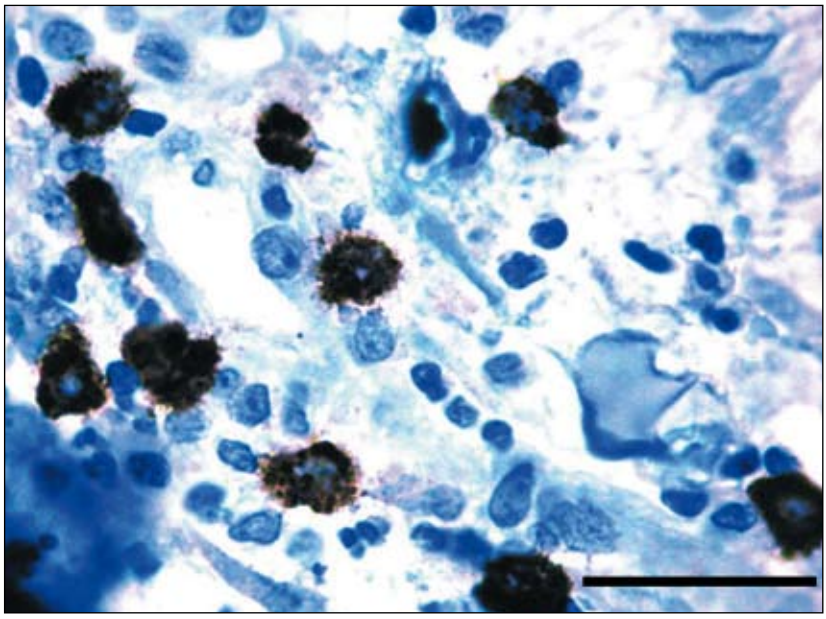

Fig. 2. Large group of mast cells in perivascular area of atopic skin, filed with secretory granules obscuring the cell nuclei. Some granules are also outside the cells borders suggestive of anaphylactic degranulation (immunoperoxidase staining, bar = 40 μm)

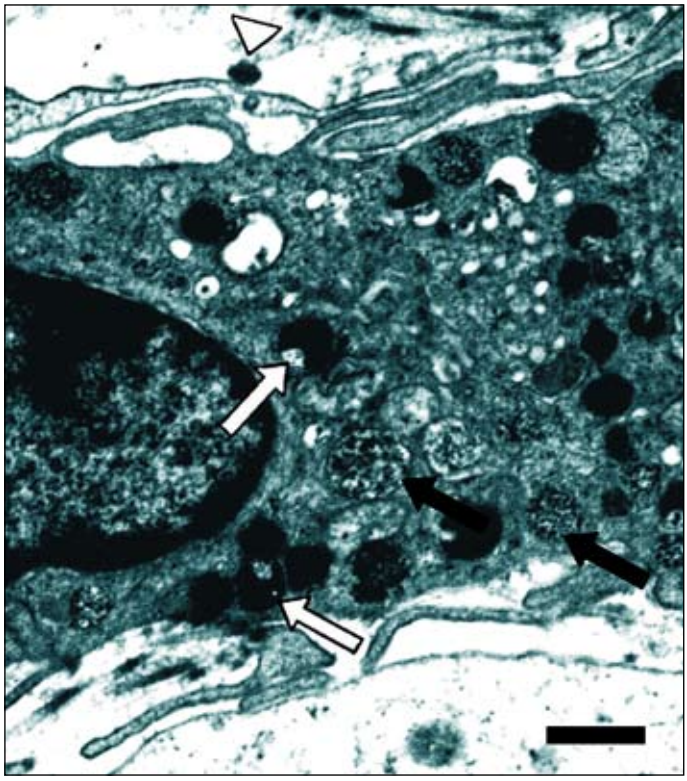

Fig. 3. Electron micrograph showing canine mast cell from atopic skin with ongoing degranulation process. Numerous oval to round granules with cores of various electron densities are visible in the cytoplasm, some of the granules are filled with a highly electron-dense homogenous material, some are more electrolucent and contain granular matrix (black arrows). Some granules containers are characterized by partially eroded cores (white arrows). All these features are suggestive of piecemeal degranulation. There are also some features of anaphylactic degranulation; extrusion of free granule (white arrowhead) trough pore in plasma membrane to the exterior of the cell (bar = 0.7 μm)