Electronic Journal of Polish Agricultural Universities (EJPAU) founded by all Polish Agriculture Universities presents original papers and review articles relevant to all aspects of agricultural sciences. It is target for persons working both in science and industry, regulatory agencies or teaching in agricultural sector. Covered by IFIS Publishing (Food Science and Technology Abstracts), ELSEVIER Science - Food Science and Technology Program, CAS USA (Chemical Abstracts), CABI Publishing UK and ALPSP (Association of Learned and Professional Society Publisher - full membership). Presented in the Master List of Thomson ISI.

ELECTRONIC JOURNAL OF POLISH AGRICULTURAL UNIVERSITIES

2012
Volume 15
Issue 1
Topic
VETERINARY MEDICINE


KALIŃSKI K., MARYCZ K., CZOGAŁA J., SERWA E., 2012. MAST CELL IDENTIFICATION AND VISUALIZATION BY MEANS OF ELECTRON MICROSCOPY, IMMUNOHISTOCHEMISTRY AND SPECIAL STAINING METHODS IN THE COURSE OF CANINE ATOPIC DERMATITIS, EJPAU, 15(1), #03.

Available Online http://www.ejpau.media.pl

# MAST CELL IDENTIFICATION AND VISUALIZATION BY MEANS OF ELECTRON MICROSCOPY, IMMUNOHISTOCHEMISTRY AND SPECIAL STAINING METHODS IN THE COURSE OF CANINE ATOPIC DERMATITIS

Krzysztof Kaliński[1,2] Krzysztof Marycz[1,2], Joanna Czogała[1], Ewa Serwa[1]

[1]*Wrocław University of Environmental and Life Sciences, Electron Microscope Laboratory, Poland*

[2]*Wrocław University of Environmental and Life Sciences, Department of Environment Hygiene and Animal Welfare, Poland*

## ABSTRACT

Atopic dermatitis (A D) is a chronic and recurring inflammatory disease with characteristic clinical presentation and significant levels of pruritus. Up to 10% of the canine population is affected atopic dermatitis. Despite many years of scientific research, there are still many of aspects of atopic pathogenesis which require further explanation. The aim of the study was the attempt to present the possibility of displaying mast cell populations in canine atopic dermatitis by using different staining protocols and microscopy techniques. The study was conducted on biopsy material collected from two groups of dogs: atopic and healthy controls. The material was tested by means of light microscopy. Additional study was conducted using TEM - Transmission Electron Microscope. A range of staining methods, as well as possible use of different microscopes types in order to identify and evaluate mast cell population in canine atopic skin, are presented and compared. Depending on the technique used it is possible to evaluate numbers, topography and activity of mast cells in canine skin.

**Key words:** mast cells, canine atopic dermatitis, dogs, TEM, AD

## INTRODUCTION

Atopic dermatitis *(łac. dermatitis atopica, AD)* is a chronic and recurring inflammatory disease with characteristic clinical presentation and significant levels of pruritus [2]. Up to 10 % of the canine population is affected atopy [10]. The first time thise disease was described in 1869 by Nalletshipa and Taya, who characterized it as a “rare hives

variety". In 1941 the atopy was confirmed in a canine patient for the first time. In 1959 Patterson presented so-called seasonal complex disease, connected with pruritus and rinitis in dogs [5]. In the 1970-80s intradermal tests were popularized which facilitated the recognition of atopy in dogs. In 1984 Willemse described this disease entity on a clinical and epidemiological basis [23]. Atopic dermatitis is counted among those diseases connected with a type I hypersensitivity reaction with a particularly strongly marked so-called late phase reaction (LPR). It results from a genetic predisposition to develop IgE-depended immunological response against common environmental antigens. Reaction could be caused by allergens existing in environment seasonally; for example, pollens and fungi [12]. The symptoms appear in a specific season of the year. The reaction appear also as a result of allergens permanently existing in the environment; for example, house dust mites. In the latter case symptoms remain all year round [16,15, 18, 21, 22]. Traditionally, atopic dermatitis is connected with the presence of mast cells and anaphylactic response. Apart from mast cells, other components of the skin immune system (SIS) such as epidermal dendritic cells, T cells, and keratynocytes play an important role in the course of atopic dermatitis.

Mast cells were first identified by Ehrlich, during histological examinations in 1876, and he described some cells containing granulations which were stained differently than surrounding tissues [17]. Rather than in the peripheral blood system, mast cells are mostly found in tissues of the whole body, particularly in the skin, respiratory system and alimentary system. Mast cells as immature forms leave out bone marrow and differentiate after settling the given tissue [9]. They are a part of the immunological system associated with anaphylactic response and defence against parasites. Mast cell activation results from antigen binding with the FcεRI receptor of membrane- bound IgE antibodies (allergens should possess the ability to bind at least two IgE antibodies). Then aggregation of receptors occurs. Gathered receptors cause biochemical changes which result in the process of degranulation and the release of bioactive substances such as histamine, serotonine, interleukins and some chemotactic factors [3, 6]

## MATERIALS AND METHODS

Research was conducted on dogs from the Silesia region. The animals were divided into atopic and healthy control groups with 10 individuals in each. The first group included client-owned dogs diagnosed with atopic dermatitis. The diagnosis was affected by fulfillment of the clinical criteria of this disease and carefully conducted differential diagnosis. In all atopic individuals intensive pruritus was noticed. The thorough anamnesis was carried out including environmental description, the age when the symptoms appeared, possible contact with other animals. Precise information about the feeding principles of each individual was also obtained.

Additionally as an exclusion procedure anti-scabies therapy with ivermenctin and flea treatment was performed. Skin samples were collected from atopic dogs using local anesthesia. From healthy dogs punch biopsy specimens were taken during routine surgical procedures e.g. sterilization. Those dogs with atopic dermatitis primarily manifested lesions such as papules, *erythema* and *erythematous macules*. Secondary lesions were also observed, in the form of alopecia hyperpigmentation, lichenification, excoriations and crusting lesions. Some dogs manifested changes in appearance of their hair coat such as mat-looking and excessively dry hair.

### Histological examination

Collected samples were fixed in a 10% buffered formalin and paraffin-embedded. 5 μm –thick sections were obtained on Zeiss Microm HM 340E and placed on histological slides. After that material was deparaffinized, hydrated in alcohol series and stained with combined eosinophil/mast cell stain (C.E.M.) and Toluidine Blue stain. Combined eosinophil/mast cell stain (C.E.M.) Slides were put in Astra Blue Stain for 30 minutes and then placed into running tap water. After that slides were put in Vital Red Stain, agitated several times and stained for 30 minutes. Slides were rinsed in running tap water and put in Modified Mayer's Hematoxylin for 15 minutes; then rinsed again in running tap water for 3 minutes and dehydrated. Finally, slides were coverslipped with DPX. Specimens were observed in a LE Zeiss Axio Imager.A1.

### Toluidine Blue Staining

Sections were stained with toluidine blue working solution for 2-3 minutes and washed in distilled water (3 changes). Then they were dehydrated quickly through 95% alcohol and two changes of 100% alcohol. Next the slides were cleared in xylene (2 changes 3 minutes each) and coverslipped with DPX medium. Specimens were observed in LE Zeiss Axio Imager.A1.

### Immunohistochemical examination

Monoclonal antibodies against mast cell tryptase were employed, using the Immunoperoxidase Cell Staining System. Heat induced antigen retrieval was performed. Slides were incubated in Tris/EDTA buffer (pH=9,0) for 20 min. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide for 5 min. Slides were washed with TBS for 5 minutes each then incubated with primary antibodies against mast cell tryptase (Zytomed Systems, dilution 1:10 000) for 1 hour at room temperature. After washes with three changes of TBS for 5 min each detection was performed using 3,3' diaminobenzidine substrate (Dako). Sections were after that counterstained with Mayer's

haematoxylin and dehydrated through alcohols and xylenes. Slides were coverslipped with DPX medium and observed via light microscopy (Zeiss, Axio Imager A1).

**Ultrastructural examination**

Collected samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After several rinses in the same buffer the material was post-fixed for 2 hours in 2% osmium tetroxide in the buffer. Following dehydratation in an acetone series (30-100%), the material was embedded in Epon 812. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined via transmission electron microscopy (Tesla BS500).

## RESULTS

In this study toluidine blue, combined eosinophil/mast cell stain (C.E.M.) (Fig.a and b). and immunostaining with monoclonal antibodies against mast cell tryptase were used (Fig. e and f).. Additionally ultrastructural analysis was performed by means of transmission electron microscopy(Fig. g and h). Toluidine blue staining method resulted in mast cell violet/red to purple secretory granules staining(Fig. c and d). The background as a orthochromatic area stains blue. Part of slides was stained with combined eosinophil/mast cell staining C.E.M. Mast cells have been stained bright blue, eosinophiles were visible as distinct cells with red cytoplasm and blue nuclei while the surrounding tissue was almost clear. Immunohistochemical staining with antibodies against mast cell tryptase was strongly positive and resulted in dark brown staining of secretory granules content within examined skin. Upon electron microscopic examination some mast cells from the perivascular and subepidermal area of the atopic skin displayed secretory granule complement with relatively high, homogenous granule electrodensity. Part of the mast cells contained granules of different electrodensities namely electrolucent, electrodence and partially empty. In some cases free secretory granules were visible outside the cellular space of the given mast cell. Comparison and characterization of presented methods in Table 1.

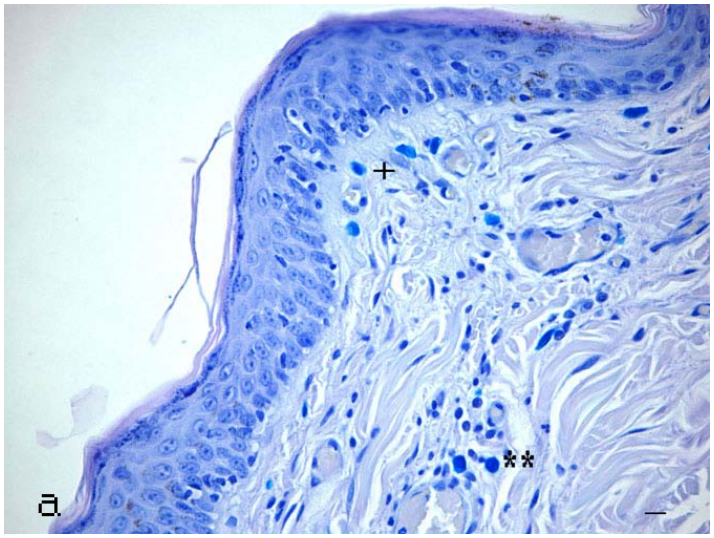



Fig.a. Atopic skin from the abdominal area of the dog stained with C.E.M. staining. Note moderate hyperplasia of the epidermis(*), and mild hyperkeratosis. In both, the subepidermal (+) and perivascular region of the skin (**) mast cell and lymphocyte infiltration is observed. Scale bar 20µm.

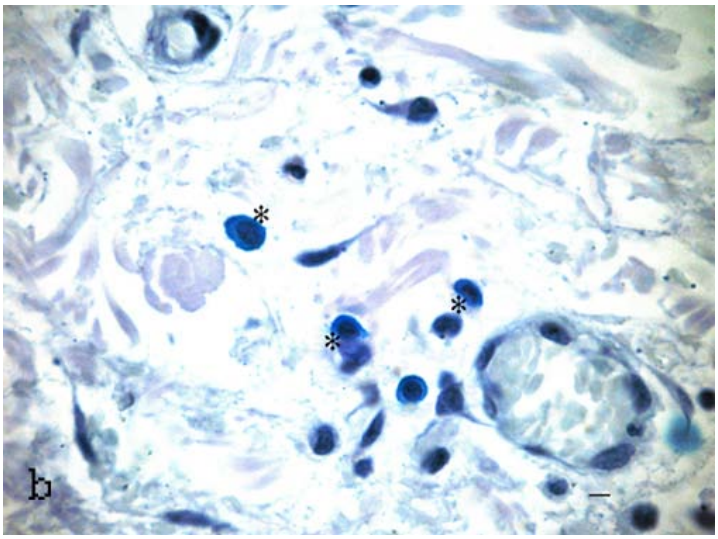



Fig.b. The perivascular region of the atopic dog skin with mast cell reach inflammatory infiltrate. C.E.M. staining ( * ). Scale bar 20μm

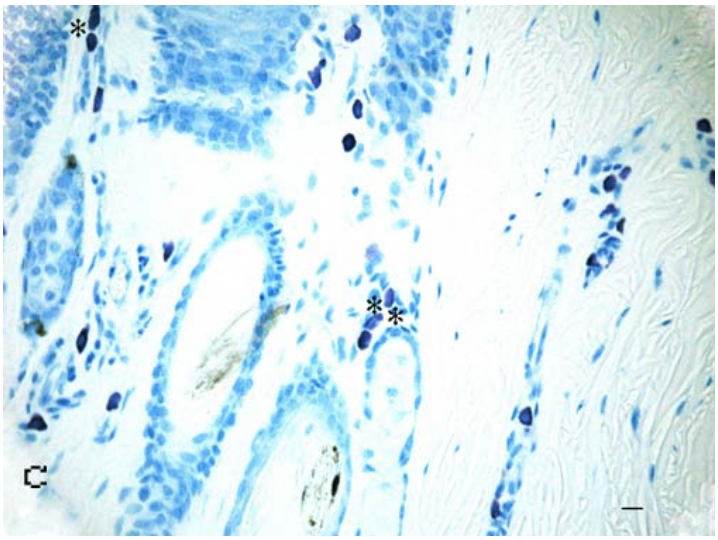



Fig. c. Abundant mast cell population accumulating in diverse regions of the skin: under epidermis ( * ), in the perivascular region and in periadnexal (**) localization. Scale bar 20μm

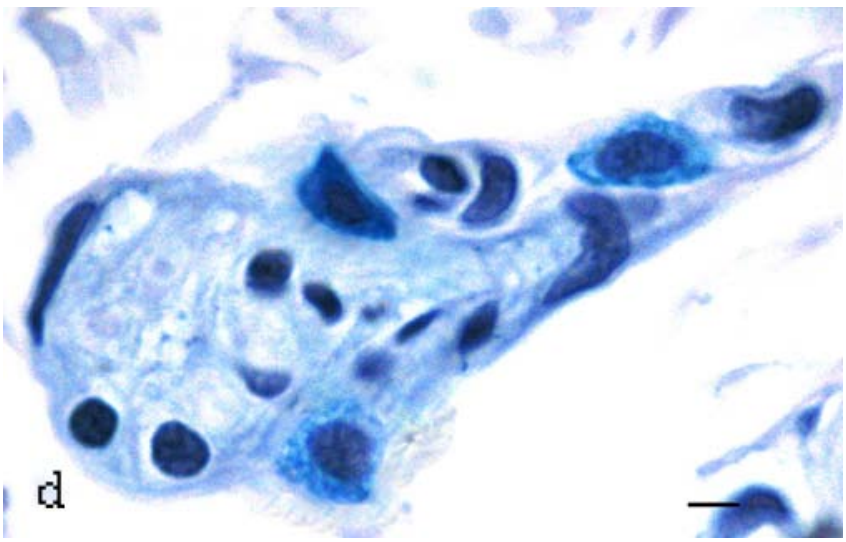



Fig.d. Perivascular region of the skin of the dog with atopic dermatitis stained with toluidin blue. Note mast cells accumulating in the close proximity of poscapillary vessel and activated endothelial cells within the vessel wall. Scale bar 10μm

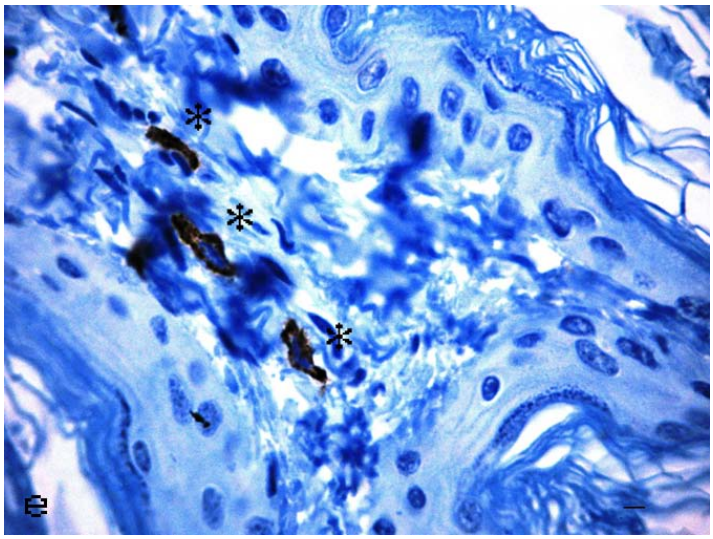
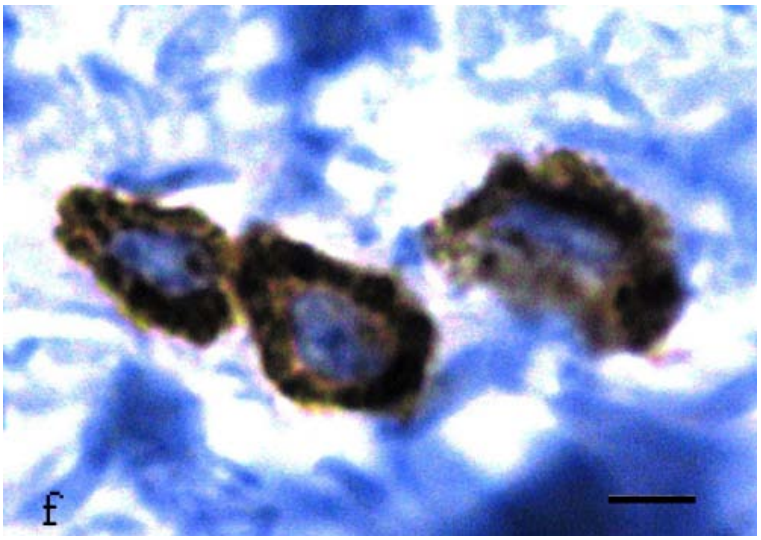


Fig.e. Immunohistochemically stained mast cell group in (*) the subepidermal compartment of the abdominal skin. Scale bar 5µm



Fot f. Photomicrograph of the canine atopic skin stained for mast cell tryptase; well preserved topography of secretory granules within individual mast cell. Scale bar 10µm

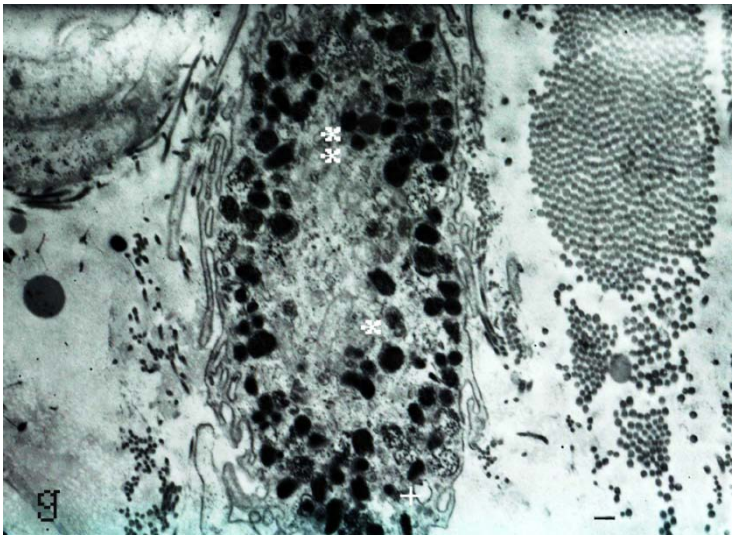


Fig.g. Mast cell showing piece meal type of degranulation. Accumulation of secretory granules in marginal compartments of the cell. Different granular patterns of different electron density are seen: dark granules of very high density (**), light granules (*), almost empty granules with remains of material electron dense material (+). Scale bar 2µm

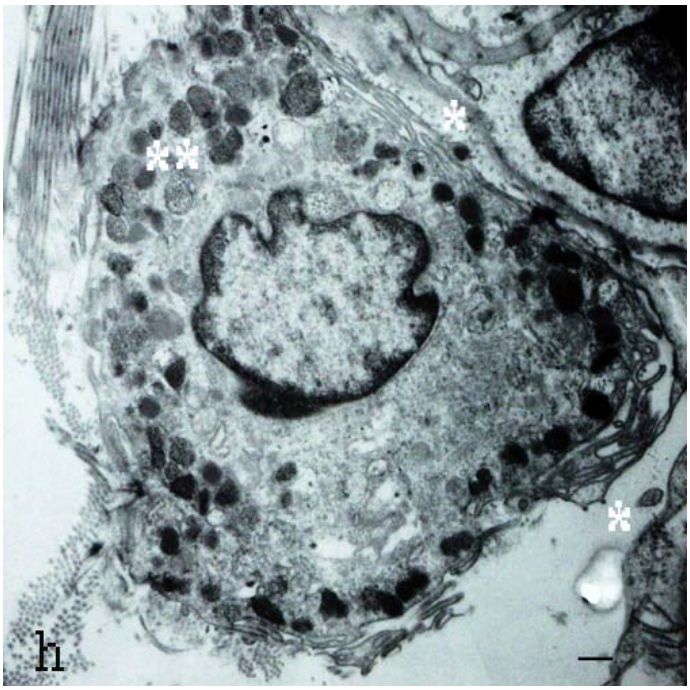



Fig.h. Mast cell showing simultaneously anaphylactic and mixed type of degranulation. Outside the cellular space of the mast cell there are free secretory granules visible (*). Also we can see fragmentary losses of granules, characteristic for piece meal degranulation(PMD). (**). Scale bar 2μm

Table 1. Comparison and characterization of present methods

| method | C.E.M. | Toluidine Blue Staining | Mast Cell Tryptase | TEM |
|---|---|---|---|---|
| Nucleus | +/- | +/- | +/- | + |
| Secretory granules | + | + | + | + |
| Type of degranulation | - | - | +/- | + |

## DISCUSSION

In the following study both light microscopy and electron microscopy techniques were used to evaluate mast cell population in the skin of atopic dogs. Moreover, diverse staining methods were compared with regards to their mast cell visualization efficiency.

Mast cells are a key factor in the inflammatory and allergic cascade during the course of atopic dermatitis both in dogs and human beings [6, 15]. The most prominent and at the same time the most characteristic, morphological feature of mast cells are their secretory granules. They contain significant levels of biologically active substances such as serine proteases, histamine, interleukins, chemokines, heparin and chondroitin sulfate [7]. In this study toluidine blue staining as a selective stain for mast cells in tissue sections was presented. The aforementioned staining method is based on metachromasia phenomena and allows to prominent mast cell secretory granules staining due to heparin and chondroitin sulfate granule contents.. Detection of mast cells with the letter staining method seems to be very effective (Fig. c and d). This technique allows to precisely quantitatively describe mast cells deployed in the given tissue. Mast cell and also eosinophiles are crucial cellular components of canine and human atopic dermatitis [14]. Simultaneous detection of mast cells and eosinophiles in different tissues including skin has been problematic. This is mainly connected with differing fixation methods preferable for mast cells and eosinophiles respectively. Combined eosinophil/mast cell staining C.E.M. implied in the following study serves to allow concurrent visualization of mast cells and eosinophiles in paraffin sections. In C.E.M staining mercuric

chloride-tannic acid as fixative and Astra Blue as staining factor allowed mast cells and eosinophiles to be successfully detected within canine atopic skin (Fig.a and b).

The previously mentioned methods do not permit precise description of the mast cell secretory granule topography and only partially defined their biochemical content. Those features may be fulfilled to a significant extent by immunohistochemical techniques. Immunohistochemistry is the process of localizing a given element in a tissue exploiting the principle of antibodies binding specifically to their given antigens. Based on morphology and immunohistochemistry three types of mast cells are distinguished [19, 8, 9]. Type one, the most common, is predominant in the skin, mainly contains vesicles with amorphic electron dense content. This type synthesizes tryptase and chymase. The second type, less common, predominant in the lungs, shows mainly vesicles with scroll-like content and osmiophilic lipid droplets of varying electron density. This type contains only tryptase. The third type of mast cell synthesizes only chymase. Antibodies applied against mast cell tryptase created the possibility to show not only the presence of tryptase containing mast cells within canine skin but also display secretory granule distribution patterns. Based on results obtained the immunostating technique seems to be most comprehensive procedure as far as mast cell identification possibilities are concerned (Fig. e and f). Transmission electron microscope examination, although not available on an everyday basis, may serve as a precise and valuable technique. With the aid of transmission electron microscopy the identification of not only ultrastructural properties but also partly functional characteristics of mast cells was possible (Fig. g and h). Mast cells are ubiquitous cells of the connective tissue. Their cytoplasm contains several hundred secretory vesicles. Stimulation by immunoglobulin E (IgE) dependent mechanisms results in intact vesicles leaving the cell and also vesicle fusion with the cell membrane and release of vesicle content. This immediate release of mast cell granules has been called compound (anaphylactic) degranulation which is of major importance in allergic reactions of the immediate type (Type 1 hypersensivity). Besides anaphylactic degranulation, there may also be a much slower granule release mode, termed piecemeal degranulation [20, 4]. Mast cells from the perivascular and subepidermal area of the atopic skin which displayed secretory granule complement with relatively high, homogenous granule electrodensity may correspond with the resting cell stage. Mast cells containing granules of different electrodensities could represent piece meal or mixed degranulation pattern. Degranulation pattern connected with the presence of free secretory granules outside the cell borders is defined as anaphylactic type. Piece meal degranulation (PMD) as a model for chronic inflammatory diseases dominated in the case of atopic dogs while the anaphylactic type was less frequent applying different microscope types and staining methods to evaluate mast cell population seems to be an interesting comparative examination. Depending on the technique used it is possible to evaluate numbers, topography and activity of mast cells in canine skin with atopic dermatitis. Among all the techniques tested transmission electron microscopy gave the most precise images of mast cells including cell morphology, granule topography and mode of secretory granule release.

## REFERENCES


1. Day, M.J., 2006. An Overview of the Skin Immune System, ESVD Workshop on Cutaneous Immunology, Coombe Lodge, Blagdon, Bristol, 28.2-2.03.2006, pp.3-9.
2. Day, M.J., 2006. Immunodiagnostic testing: Serological Tests, ESVD Workshop on Cutaneous Immunology, Lodge, Blagdon, Bristol, 28.2-2.03.2006, pp. 93-100.
3. Day, M.J., 2005. Immunomodulatory therapy, [in:] Hillier A., Foster A. P. (edit.), Advances in Veterinary Dermatology 5.
4. Dvorak, A.M., 2005. Ultrastructural Studies of Human Basophils and Mast Cells. J. Histochem Cytochem 53,1043–1070
5. Griffin, C.E., DeBoer D.J., 2001. The ACVD task force on canine atopic dermatitis (XIV): clinical manifestations of canine atopic dermatitis. Veterinary Immunology and Immunopathology Volume 81, Issues 3-4, Pages 255-269
6. Gołąb, J., Jakóbisiak, M., Lasek, W., Stokłosa, T., 2007. Immunologia. PWN, Warszawa.
7. Gurisha M.F., Austen F.K., 2001. The Diverse Roles of Mast Cells. J. Exp. Med., 194(1) f1–f6.
8. Hill, P.B., 2000. Mast cells: a review of their biology and role in cutaneous inflammation, [in:] Thoday K. L., Foil C. S. (red), Bond R. (edit.), Advances in Veterinary Dermatology: Proceedings of the Fourth World Congress of Veterinary Dermatology, San Francisco, California, USA,
9. Kuryszko, J., Zarzycki, J., 2000. Histologia zwierząt. Warszawa.
10. Marsella, R., Nicklin C.F., 2001. Sulphido-leukotriene production from peripheral leukocytes and skin in clinically normal dogs and house dust mite positive atopic dogs. Vet. Dermatol. 12, 1, pp. 3-12.
11. Olivry, T., Moore P.F., Affolter V.K., Naydan D.K., 1996. Langerhans cell hyperplasia and IgE expression in canine atopic dermatitis. Archiv. Derm. Res., 288, pp. 579-85.
12. Olivry, T., Foster P., Mueller, S., McEwan A.N., Chesney C., Williams, C.H., 2010. Interventions for atopic dermatitis in dogs a systematic review of randomized controlled trials. Veterinary Dermatology, Volume 21, pp. 4-22(19)
13. Patterson, R., 1960. Investigations of spontaneous hypersensitivity of the dog. J. Allergy 31, pp. 351–363.
14. Piliponskya, A.M., Pickholtza D, Gleichb, J. Gerald, J., Levi–Schaffera F., 1999. Eosinophils Activate Mast Cells to Release Histamine. Int. Arch. Allergy Immunol.,118,202-203
15. Pomorski, Z.J.H., 1990. Alergeny wziewne a rozwój występujących sezonowo oraz utrzymujących się stale zmian skórnych typu egzema atopia u psów. Ann. UMCS. Lublin-Polonia, 45, 75-85
16. Ready, L.M., Miller, W.H., Willemse, T., 1997. Alergic Skin Disease of Dogs and Cats. Caunders Company.

17. Sapieżyński, R., Sapieżyńska, E., 2005. Nowotwory skóry i tkanki podskórnej u psów i kotów. Część I Guzy komórek tucznych. Życie weterynaryjne 80(5) 283-88
18. Scott, D.W., Miller. W.H., Grinffin, C.E., 2001. Small Animal Dermatology, Muller&Krik's W.B Sanuders Company.
19. Sell, S., 1987. Immunology, Immunopathology, and Immunity. New York-Amsterdam-London
20. Shin Joon., Kang Ho Suck., Chung In Hyuk., Lew Jae Duk., Lee Young Don., Cho Yyung Pil., Shin Tai Sun 1986. An Electron Microscopy Study on Mast Cell Degranulation and Regeneration in Rats. Yonsei Medical Journal Vol.27, No.1,
21. Szczepanik, M., Wilkołak, P., Taszkun, L., Pomorski, Z., 2005. Atopowe zapalenie skóry psów w świetle alergenów odpowiedzialnych za rozwój uczulenia. Medycyna Wet. 61. 305-308.
22. Taszkun, I., Śmiech, A., 2006. Zmiany histopatologiczne skóry w chorobach alergicznych u psów. Życie Weterynaryjne 8,538-43.
23. Willemse, A., 1986. Atopic skin disease: a review and a re-consideration of diagnostic criteria. J. Sm. Anim. Pract. 27, pp. 771–778.

---

Krzysztof Kaliński
Department of Animal Hygiene and Animal Welfare, Electron Microscope Laboratory,
Wrocław University of Environmental and Life Sciences, Poland
Kożuchowska 5b, 51-631 Wrocław, Poland
Phone: +48 71 320 58 88
email: krzysiekkalinski@gmail.com

Krzysztof Marycz
Department of Animal Hygiene and Animal Welfare, Electron Microscope Laboratory,
Wrocław University of Environmental and Life Sciences, Poland
Kożuchowska 5b, 51-631 Wrocław, Poland
Phone: +48 71 320 58 88

Joanna Czogała
Electron Microscope Laboratory, Wrocław University of Environmental and Life Sciences, Poland
Kożuchowska 5b, 51-631 Wrocław, Poland
Phone: +48 71 320 58 88

Ewa Serwa
Electron Microscope Laboratory, Wrocław University of Environmental and Life Sciences, Poland
Kożuchowska 5b, 51-631 Wrocław, Poland
Phone: +48 71 320 58 88

---

Accepted for print: 3.02.2012