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Mast Cell Morphological Heterogeneity: An Indicator of Skin Immune System Activation in Horses Affected with Summer Eczema

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Abstract: Thirteen, 7 months old thoroughbred horses with no clinical signs of insect bite hypersensitivity, commonly exposed to culicoides bites were studied. Allergic individuals were determined by a positive intradermal skin test, performed with culicoides allergen. Animals with negative skin test served as a control. In horses serum levels of IgG were examined. From horses with positive skin test results, skin biopsies were examined by means of light and electron microscopy. Positive reaction against culicoides allergens confirmed that IgG even in absence of IgE can mediate allergic response in <1 year old horses. Ultrastructural skin studies showed marked activation within mast cells population in allergic horses even though they exhibited no clinical symptoms of summer eczema. There was marked domination of piece meal type of degranulation observed which indicated persistent inflammatory process within skin of asymptomatic animals. Moreover, skin mast cells of allergic horses whose IgG serum levels were relatively high besides piece meal often manifested anaphylactic and mixed type of degranulation.

Key words: Sweet itch, equine, mastocyte, degranulation, allergy

INTRODUCTION

Sweet itch (Summer eczema) is the form of Insect-Bite Hypersensitivity (IBH) caused by culicoides allergens in horses (Marti *et al*., 2007; Hamza *et al*., 2008). About 30% of different horse breeds worldwide is IBH affected. Iceland where culicoides midges are absent is one of the rare countries (Kolm *et al*., 2006). The most characteristic clinical feature of the disease is pruritus and seasonality from spring until autumn (Cunningham and Dunkel, 2008). Mainly 1 year old horses or older are affected. The allergens causing IBH are salivary gland proteins from culicoides midges (Hamza *et al*., 2008; Walsh, 2003). In the insects' saliva, >10 proteins with the ability to bind IgE have been found (Hellberg *et al*., 2006). Recent investigations allowed to identify several culicoides saliva allergens like proteins involved in sugarmeal digestion, defense and anti-coagulation (Walsh, 2003). A type I hypersensitivity reaction connected with mast cell degranulation is considered to be the main mechanism of acute phases of IBH whereas in chronic stages type IV hypersensitivity pattern could be also involved (Mckelvie *et al*., 2001; Cunningham and Dunkel, 2008). Sera of IBH-affected horses contain IgE and IgG (T) which sensitize mast cells (Schaffartzik *et al*., 2009). Furthermore, IgG (T) antibodies are supposed to influence the early phases of allergy development which is particularly important in young horses before they are able to develop production of allergen specific IgE (Wagner, 2006; Wagner *et al*., 2008). Equine serum IgE transferred with colostrum disappeared from the circulation within the first 3-4 months of age. The endogenous IgE production begins at 9-11 months of age. The late onset of endogenous IgE production explains why IgE mediated

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allergies are generally not observed in horses before puberty (Wagner, 2006; Langner *et al*., 2009). On the other hand, it was indicated that not only allergen-specific IgE but also IgG are responsible for hypersensitivity reactions in horses (Wagner, 2006). Equine IgG (T) can bind to high-affinity FcεRI receptor on equine skin mast cells and can activate degranulation (Wagner *et al*., 2008). For this reason analysis of skin biopsies and IgG serum levels of 7 months old horses reacting positively to intradermal injected culicoides allergens seems to be an interesting research model as far as Summer eczema of young horses is concerned.

A typical role of mast cells is traditionally connected with anaphylactic reactions. At present mast cells, besides T lymphocytes are considered to be the major effector cells in the course of many allergic conditions being responsible for the immune cells infiltration into target tissues. In the recent years, investigators have focused on the role that the mast cells play in the induction phase of allergic diseases through their immunomodulation properties. Depending on the kind of stimuli, some environmental factors and heterogeneity among responding mast cells granules either Piece Meal (PMD) or Anaphylactic Degranulation (AND) occurs. Sometimes different deregulation patterns undergo within the same mast cell. PMD dominates in the course of certain chronic diseases like bullous pemphigoid, contact dermatitis, atopy and during wound healing. Monocyte Chemotactic Protein-1 (MCP-1) is the most potent histamine-relasing chemokine associated with PMD. Effective triggers for AND are IgE, anti-IgE, opiates, calcium ionophore, compound 48/80 (Dvorak and Kissell, 1991; Dvorak, 2005; Wagner, 2006). The exact functional significance of different degranulation patterns isn't defined yet but such distinct modes of degranulation would agree with diverse biological roles of mast cells population. In view of above mentioned observations the purpose of this study was to examine mast cells population in horses <1 year with no clinical symptoms of IBH diagnosed as being allergic on the basis of intradermal test. Moreover, in current research, researchers made attempt to find connections between histopathological and ultrastructural picture of the skin mast cells and IgG serum levels in examined horse group.

MATERIALS AND METHODS

Horses: Thirteen 7 months old, thoroughbred horses both sexes were encountered into this research. The animal group chosen showed no clinical signs of insect bite hypersensitivity and they exhibited no other symptoms of any disease. All horses were kept in Golejewko in central-West part of Poland. The stable is situated in the close proximity to several lakes with standing water. The animals were kept in a stable with an access to open paddock and meadow spending the majority of the day outside being exposed to culicoides bites. At first the blood was collected then intradermal tests performed and finally skin was biopsied from examined horses in mentioned order.

Collection of peripheral blood and ELISA IgG testing: From the horse population examined (13 individuals) samples of peripheral blood were collected into EDTA tubes by jugular venipuncture and the level of IgG antibodies were examined by means of The IMMUNO-TEK Horse IgG ELISA kit (ZeptoMetrix).

Intradermal skin testing: Horses were clipped at the lateral neck and the Culicoides allergen extract was injected intradermally at 1000 PNU mL^{-1} dilution into the left site of the neck approximately 10 cm below the central mane line. Histamine and saline (both Greer Laboratories, Lenoir, NC) were used as positive and negative controls, respectively. The reaction of the skin was examined 15 min, 30 min and 24 h later. Based on the size of the guaddel , a score (0-4) was applied to each injection site. A score of 0 was given for wheals equivalent to those seen for the negative control and a score of 4 was given to wheals seen for the positive control. A score of 2 was given to wheals that were intermediate between the two controls. Wheals with a score of 2 or greater were considered positive reactions. About 9 out of 13 examined horses had positive test results 30 min after intradermal injection and were considered to be allergic. Animals with negative results were treated as negative control.

Skin biopsy: About 7 mm skin biopsies were taken using local anesthesia (lidokaine injectable 2%) from the right side of the neck from horses with positive reaction during intradermal skin test. Each specimen was fixed immediately after sampling. One half of the specimen was fixed in a 4% neutral buffered formalin and later, paraffin embedded 5 μm sections were stained with C.E.M. stain kit (dbs) for mast cells and eosinophils identification. The second half of each specimen was fixed in 3.5% glutaraldehyde in a phosphatic buffer (ph 7, 2-7, 4). Fixed tissues were then embedded in epoxy resin (Epon 812) for the purpose of a transmission electron microscopy examination (Tesla BS500). About 70 nm sections were stained with uranyl acetate and lead citrate. About 2 μm section were stained with 1.0% toluidine blue.

Statistical analysis of the results: Student's t-test was performed to compare IgG serum levels in control and allergic horses.

RESULTS AND DISCUSSION

Intradermal skin test revealed that majority of the horses (9 out of 13) reacted positively 30 min after intradermal injection of culicoides extract which was manifested by significant wheal formation in the injection site. The concentration of IgG in the horse serum ranged in allergic horses from 43.0 ng mL^{-1} up to 65.625 ng mL^{-1} while in non allergic animals from 39.1-41.8 ng mL^{-1}. Student's t-test evaluation revealed no statistically significant differences between allergic and control horses as far as IgG serum level is concerned. In histological cross-sections of the skin derived from allergic individuals the intensity of inflammatory infiltrates were mild to moderate. Histological examination indicated the presence of prominent polymorphous inflammatory cell infiltrates in the subepidermal and particularly in perivascular compartment of the skin. Mast cells, granulocytes and fibroblasts were main cellular components of the inflammatory infiltrates. In some skin areas the pattern of inflammation progressed from the perivascular to the interstitial one. Epidermis in majority of allergic horses demonstrated the features of hyperpigmentation. The observed abundant melanocytes had characteristic appearance of clear cells and were found mainly among the basal cell layer (Fig. 1) but they were also observed in the follicular compartment. Applied CEM staining allowed to demonstrating simultaneously eosinophils and mast cells in examined horse skin (Fig. 2). They were present mainly in two compartments of the skin: near the epidermis and in perivascular area and could be observed as individual cells as well as cell clusters. Mast cells were visible as bright blue cells with dark blue nucleus, eosinophils as bright red cells with visible granules and blue nucleus. Electron microscopic studies of mast cell population revealed distinct features of granules electrodensity and different granular patterns. Furthermore, variable numbers of secretory granules and different mode of their distribution within individual cells was observed. Numerous oval to round granules with cores of electron densities ranging from dark to almost electron lucent were visible in the cytoplasm. Some of the granules were filled with a highly electron-dense homogenous material, others contained mixed or particle type of granular matrix. The electron microscopic examination of the mast cells in allergic horses revealed that they undergo different types of ultrastructural alternations of secretory granules namely piece meal and anaphylactic pattern of degranulation. In examined skin of allergic horses with relatively high IgG serum levels mast cells manifested often ultrastructural features of anaphylactic degranulation and mixed type degranulation.

One can appreciate the degranulation chambers and secretory granules or their fragments outside of the cell borders (Fig. 3 and 4). Mast cells of allergic horses with lower serum IgG level also exhibited significant degranulation activity but mainly of PMD pattern. Granules piecemeal losses were focal from single granules or had features of complete losses of single granules contents from variable numbers of secretory granules. One could notice, typical of PMD, slightly enlarged, non-fused, empty and partially empty granule containers in undamaged mast cells (Fig. 5). Mast cells in described equine group relatively often showed the features of recovery from degranulation process even within otherwise granulated cells. Ultrastructural studies

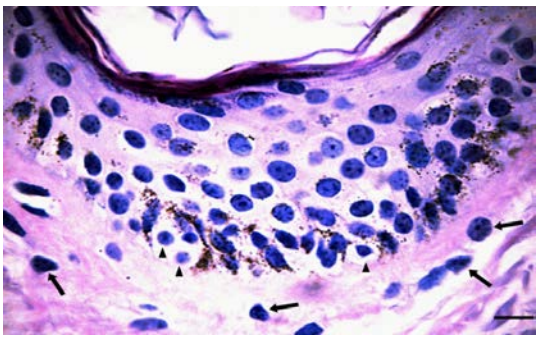

Fig. 1: Equine epidermis and subepidermal compartment of the skin. The observed abundant melanocytes had characteristic appearance of a clear cells (black arrow heads) and are found mainly among the basal cell layer. Granulocytes are visible in subepidermal compartment (black arrows) (CEM staining, bar = 50 μm, 40x)

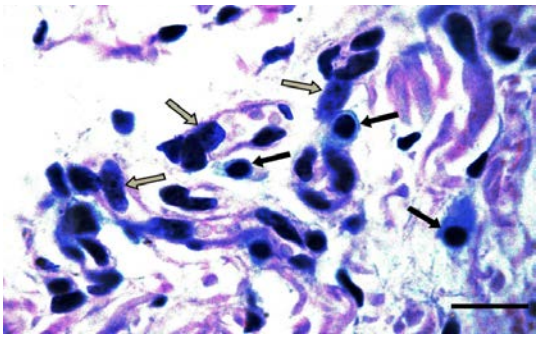

Fig. 2: Photomicrograph of equine skin. Mast cells (black arrow) in biopsy specimen derived from perivascular area can be found in the close proximity to eosinophils (white arrow) and other cells of the inflammatory infiltrate (CEM staining, bar = 25 μm, 100x)

Fig. 3: Electron micrograph showing a degranulated mast cell from the perivascular region of equine skin. There are degranulation empty chambers visible in cell cytoplasm (open arrow) as well as extruded secretory granule outside the cells border (black arrow) (bar = 1.8 μm, 14000x)

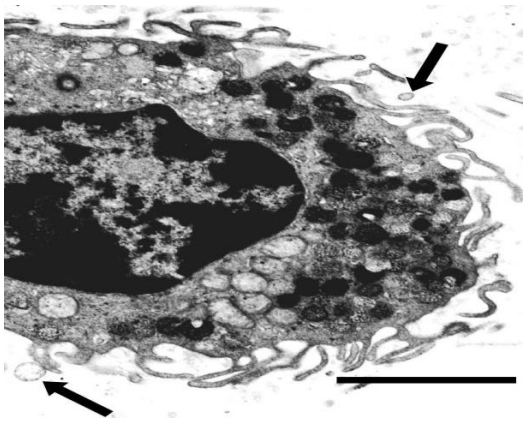

Fig. 4: Electron micrograph showing equine skin mast cell with ongoing PMD. There are also some features of AND within described cell: extrusion of altered membrane free granule (black arrows) through pore in plasma membrane to the exterior of the cell borders is observed (bar = 0.7 μm)

revealed canalicular structures found in peripheral cytoplasmic areas and condensation of denser matrix material centrally or at the margins of lucent granule chambers (Fig. 6).

Summer eczema is common disease of horses with known etiological agent and well described clinical picture. Still there are some areas of pathogenesis of equine IBD where the knowledge is not complete. The pivotal role of IgE in immediate skin reactions in horses is indisputable but there are only few works describing the IgG class significance in the course of equine summer eczema. Confirming hypothesis that allergic response in horses can be mediated by IgG seems to bee particularly

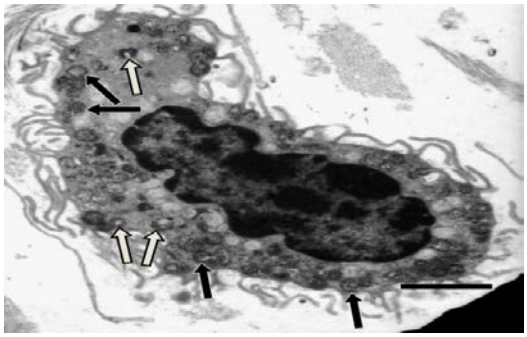

Fig. 5: Electron micrograph showing equine skin mast cell with ongoing piece meal degranulation process. Numerous oval to round granules with cores of various electron densities can be seen in the cytoplasm; some of the granules are more electrolucent and contain particle matrix (solid arrows) some are slightly dilated, nonfused, empty or with partially eroded cores (open arrow) (bar = 1.2 μm, 18000x)

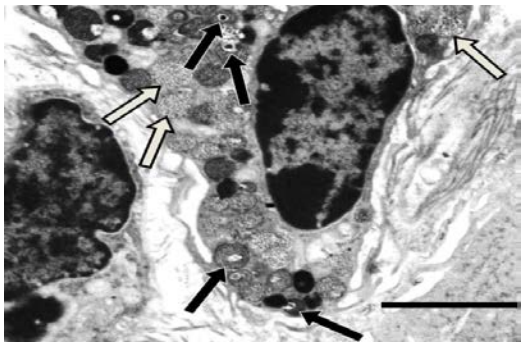

Fig. 6: Electron MC micrograph showing ultrastructural features suggestive for PMD and recovering from PMD process. Observed granule electron densities ranging from dark to almost electron lucent. There are some granules with mixed and particle (open arrow) granular patterns. Some of cytoplasmic granules displayed focal areas of condensation of dense material central or peripheral in locations (black arrows) which is characteristic for recovery process (bar = 1 μm, 24000x)

arguable in young animals because of their particular immunoglobulin's serum profile basically lacking IgE class.

During this study, researchers besides analyzing IgG serum levels had also examined the histopathological picture of the skin of allergic horses. The subject of particular interest was mast cell population as a key regulatory and effector cell of the skin hypersensitivity

reactions. Histamine, eicosanoids and cytokines released after mast cell degranulation trigger the inflammatory reaction (Scott and Miller, 2003). Histopathological picture of natural disease involves eosinophilic and/or lymphocytic superficial to deep perivascular dermatitis or eosinophilic or lymphocytic vasiculitis. Acute IBH lesions are characterized by cutaneous oedema, infiltration with eosinophils, increased numbers of IgE-bearing mast cells and increased expression of Th2 cytokines. Chronic lesions are characterized by usually marked hyperkeratosis and T cell infiltrates. A similar histopathological picture of equine skin affected with IBD was observed in current research despite no clinical signs of disease were noticed. Eosinophils which were observed in the skin of experimental allergic horses in high numbers are considered to be important effector cells in equine sweet itch. Release of eosinophil-derived products such as superoxide anions, granule enzymes, lipid mediators could cause local tissue damage and further recruitment of inflammatory cells (Marti *et al*., 2007, 2009).

Despite essential role of mast cell in equine Summer eczema there is lack of detailed information concerning mast cell ultrastructural characteristics and predominant pattern of their degranulation during the course of equine IBH. In examined skin of allergic horses there was marked domination of piece meal degranulation. Mentioned above observations are consistent with other studies which provide evidence that during chronic inflammatory skin diseases, the predominance of the piece meal over anaphylactic degranulation occurs (Kaminer *et al*., 1995; Kolm *et al*., 2006). With respect to equine Summer eczema it might be explained by existence of permanent inflammatory reactions ongoing within horse skin and presents, besides allergens, IgEs and IgGs many inflammatory mediators such as complement components, cytokines and neuropeptides that can provoke the piece meal degranulation. Comparison of serological and ultrastructural results indicated correlation between serum IgG level and activation of mast cells in equine skin as far as patterns of degranulation is concerned. The higher the levels of serum IgG were the more often anaphylactic type of mast cells degranulation occurred. It could be explained by the fact that immunoglobulins are potential triggers for anaphylactic type of mast cell granules release. This finding may additionally support the theory that not only IgE but also IgG can bind to skin mast cells and contribute to allergy state particularly in horses under 1 year old. Despite the fact that the disease is quite common and frustrating both for animals and veterinary doctors there is no perfect way to treat it yet. So far, treatment consists of attempted avoidance of exposure, use of summer eczema blankets or repellents and of symptomatic treatment with corticosteroids (Cunningham and Dunkel, 2008). There are also some attempts to treat IBH using herbal medicine but published research is limited (Williams and Lamprecht, 2008). In researchers opinion diet's supplementation with polyunsaturated fatty acids might have positive anti-inflammatory and anti-allergic effect and deserve the highest attention. It was found that polyunsaturated fatty acids may induce changes in cell membrane composition what can explain their potential anti inflammatory properties (Craig *et al*., 1997; Friberg and Logas, 1999). The problem of potential curable role of feed supplements in the course of equine Summer eczema will be the subject of researchers farther investigations.

CONCLUSION

Mentioned above observations confirm that not only IgE but also IgG can bind to mast cells and considerably contribute to allergy existence in horses under 1 year old.

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