



Flea Bite Hypersensitivity: New Aspects On The Involvement Of Mast Cells

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SUMMARY

A study was performed to test the effect of sensitization to flea antigen, followed by exposure to fleas on mast cells (MCs), their subtypes, and IgE+ cells. Biopsies were taken from flea-sensitized dogs ($n = 28$) and non-sensitized dogs ($n = 5$) that had been exposed to fleas. Control groups consisted of flea-sensitized ($n = 12$) and non-sensitized dogs ($n = 9$) that were not exposed to fleas. Biopsies, taken before, 24 and 72 h after local flea exposure, were stained with haematoxylin and eosin (H&E), toluidine blue, a double labelling technique for MC chymase and tryptase and anti-IgE. An intradermal test for flea antigen was performed and serum titres of allergen-specific IgE and IgG were measured.

Significantly higher numbers ($P < 0.001$) of double labelled MCs compared to toluidine blue stained MCs were detectable in flea-sensitized dogs independent of flea exposure. In contrast, in non-sensitized dogs, the number of toluidine blue stained MCs and the number of double labelled MCs did not differ. In flea-sensitized dogs after flea exposure the percentage of C-MC was significantly increased at day 1 ($P < 0.001$) and day 3 ($P < 0.001$), whereas the percentage of TC-MCs decreased significantly at day 1 ($P < 0.001$) and day 3 ($P < 0.05$). The percentage of T-MCs decreased ($P < 0.05$ day 0 versus day 1; $P < 0.05$ day 0 versus day 3). No significant difference was detectable after toluidine blue staining and staining for IgE+ cells between the groups nor between the MC density and the number of IgE+ cells. All flea-sensitized dogs had positive skin tests to flea antigen and high serum titres of flea-specific serum IgE and IgG antibodies. In non-sensitized dogs, these results were negative. Our data provide strong evidence for an upregulation of MC proteases during the process of sensitization and a generalized selective release of mast cell tryptase after exposure to the antigen.

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KEYWORDS: Mast cells; flea bite hypersensitivity; tryptase; chymase; IgE.

INTRODUCTION

Flea bite hypersensitivity (FBH) is a common skin disorder in dogs. It plays a major role in at least 50% of all canine dermatological disorders and often coexists with other allergic conditions such as atopic dermatitis (Kwochka, 1987; Reedy & Miller, 1989). The most important factor predisposing to FBH is exposure to the causative agent (predominantly *Ctenocephalides felis felis*), which results in sensitiza-

tion and subsequent hypersensitivity. However, exposure to fleas does not always result in flea allergic dermatitis and it has been suggested that some chronically exposed dogs may develop tolerance instead of hypersensitivity (Halliwell *et al.*, 1987). Many inflammatory pathways and mediators contribute to the development of flea allergy in the dog. It has been shown that FBH involves both type I (IgE-mediated) and type IV (cell mediated) hypersensitivity reactions (Gross & Halliwell, 1985).

The role of MCs in FBH is not clear but it is well known that they are important in the immunopathology of immediate- and delayed-type hypersensitivity reactions (Welle, 1997). The immunological

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activity of MCs can be explained by the release of both preformed and newly synthesized mediators that follows their activation (Benyon, 1989; Stevens & Austen, 1989; Huntley, 1992). Although not all components of canine MC granules have been clearly defined, it is suggested that they have the same variety of mediators as other species (Gordon *et al.*, 1990). MCs exist *in vivo* and *in vitro* as a heterogeneous family of effector cells and increasing experimental data indicate that the different functions of MCs in skin physiology and pathology are partially a result of MC heterogeneity. This heterogeneity can be demonstrated as differences in histochemical, biochemical, and functional characteristics and thus MC populations have been distinguished according to these aspects (Welle, 1997). The main criterion for canine MC subtyping is the content of the MC-specific proteases chymase and tryptase. In the dog, three MC subtypes can be distinguished: MCs that contain tryptase only (T-MC), MCs that contain tryptase and chymase (TC-MC), and MCs that contain only chymase (C-MC) (Kube *et al.*, 1998). The functional role of skin tryptase and chymase is still an open question, but their abundant presence in normal skin and in different types of skin diseases suggests that these proteases promote and modulate inflammation and remodelling of extracellular matrix.

The involvement of specific MC subtypes and MC proteases in the pathogenesis of disease in man (Harvima *et al.*, 1990; Harvima *et al.*, 1993); and in dogs (Sekizawa *et al.*, 1989; Rubinstein *et al.*, 1990; Welle *et al.*, 1999a; Welle *et al.*, 1999b) underlines the importance of a study on MC heterogeneity in canine FBH. The goal of our study was to investigate the influence of sensitization to flea antigen with and without flea exposure on MCs, their subtypes, and IgE+ cells.

MATERIALS AND METHODS

We performed this study in four separate phases using a total of 16 male and 25 female Beagle dogs from the research colony from Novartis Centre de Recherche Santé Animale SA. The studies have been reviewed by an Ethics Committee according to Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985) and the Swiss Regulations on Animal Experiments (Schweizerisches Tierschutzgesetz).

The dogs were aged between one and seven years with a mean age of four years. Dogs sensitized to flea antigen and non-sensitized dogs were used. Some of

the flea-sensitized dogs were used in more than one phase.

Sensitization procedure

Adult dogs were exposed to fleas (*Ctenocephalides felis felis*) which had been bred on bovine blood at the Novartis Centre de Recherche Santé Animale SA once a week. Using an elastic belt, a cage (diameter: 4 cm) containing 30 live fleas was held in apposition to the clipped thoracic wall of the dog for 15 min. The bottom of the cage consisted of a nylon mesh through which fleas could bite. After six to nine months of weekly exposure the dogs showed positive skin tests to flea antigen. Thereafter exposure frequency was reduced to once a month until the dogs were used in the experiment. Sensitization was monitored on a regular basis by skin tests and measuring serum titres of flea antigen-specific IgE.

Biopsy sampling

To evaluate the histopathology as well as the involvement of MCs and of IgE+ cells after flea bites, punch biopsies (8 mm) were taken under local anaesthesia (Lidocain 2%, 1.5 mL; Chassot AG) before flea exposure (day 0), and 24 (day 1) and 72 h (day 3) after exposure at the site of flea bites and at a distant control site. The biopsies were taken from flea-sensitized dogs ($n = 28$) and non-sensitized dogs ($n = 5$) that were exposed to fleas. Control groups consisted of flea-sensitized dogs ($n = 12$) and non-sensitized dogs ($n = 9$) that were not exposed to fleas.

Pathological evaluation

After fixation in buffered formalin (4%) at room temperature for 24 h, the biopsies were embedded in paraffin wax and serial sections of 4 µm were cut from each tissue block. One section of each tissue sample was stained with haematoxylin and eosin (H&E), another section was stained with toluidine blue to demonstrate the sulphated acid glycosaminoglycans in the MC granules. The protease content of MCs was examined with an enzyme-immunohistochemical staining technique using a histochemical reaction for the detection of chymase activity and an immunohistochemical staining for the detection of tryptase (double labelled MCs). The specificity of these staining techniques under standardized fixation and staining conditions for the detection of canine and bovine MCs was evaluated and reported previously (Kube *et al.*, 1998; Küther *et al.*, 1998). Briefly, the enzyme cytochemical reaction for the detection of chymase was performed with a com-

mercially available detection kit (Sigma Chemicals) using naphthol-AS-D-chloroacetate as substrate. Immunohistochemistry for tryptase was done with a polyclonal rabbit anti-human skin tryptase antibody (Harvima *et al.*, 1988). The details of the method are described elsewhere (Kube *et al.*, 1998). IgE+ cells were detected with an anti-canine IgE antibody (Griot-Wenk *et al.*, 1998).

In investigating the skin samples, the following parameters were considered:

1. Histopathological evaluation of the H&E slides.
2. Average MC density/mm² following toluidine blue staining.
3. Average MC density/mm² following the enzyme-immunohistochemical double labelling technique for MC proteases.
4. Density and percentage distribution of the MC-subtypes according to protease content.
5. Average density/mm² of IgE+ cells.

The quantification of positively stained cells with a visible nucleus was performed by using a square eyepiece graticule (objective $\times 25$, eyepiece $\times 10$, 10×10 squares with a total area of 0.0169 mm^2). The total area of each section was evaluated.

Statistical analysis

Repeated measures ANOVA, Tukey-Kramer Test, Pearson's Tests for correlation, and repeated measures analysis of variance using the SAS/STAT program were applied for statistical analysis. *P* values were considered significant when < 0.05 .

Other tests

Blood samples from all dogs were collected at day 0 and day 3 of the trial. Serum titres of allergen-specific IgE and IgG for flea saliva and whole flea antigen were measured by two different laboratories (Hesca, and Imovet BG).

Intradermal skin tests were performed to establish the presence or absence of a hypersensitivity reaction. After clipping the lateral abdomen and an intravenous administration of 0.5 mL/kg Coomassie blue solution (1% w/v), 0.1 mL soluble extract of whole fleas (1:100 w/v; Pest-Ag, Novartis) and a commercially available whole flea antigen extract (1:1000 w/v; Greer Laboratories) were injected intradermally. Saline diluent served as negative and histamine phosphate (0.1 mg/mL) as positive control. Skin tests were read after 20 min for evidence of acute hypersensitivity reaction as shown by a blue erythematous wheal. The skin test sites were reexamined at 24 and 48 h for evidence of delayed hypersensitivity.

RESULTS

Histopathological examination of the skin biopsies did not reveal any pathological changes in the skin, prior to flea exposure. No differences were observed between flea-sensitized and non-sensitized dogs. At day 1 after flea exposure, the histopathological changes at the site of flea exposure were characterized by a mild to moderate superficial perivascular infiltration with MCs and eosinophils mixed with lymphocytes, histiocytes, and some neutrophils. Mild to moderate superficial dermal oedema was also present. At day 3 after flea exposure, the perivascular infiltrate was composed mainly of mononuclear cells and of MCs. Eosinophils were rarely present. Some dogs had a mild periadnexal infiltration at days 1 and 3 after flea exposure. In the majority of biopsies from the site of flea exposure, intraepidermal eosinophilic pustules were present at days 1 and 3 (Fig. 1). At the control sites, no histopathological changes were observed. As in the biopsies taken before flea exposure, sensitization to flea antigen had no perceived histopathological effects.

With regard to MC density in the skin biopsies, no statistically significant differences were detectable after toluidine blue staining between the four groups of dogs (data not shown).

Similar results were obtained with IgE staining. No significant changes in the density of IgE+ cells were found between the four groups of dogs. Furthermore, within each individual group of dogs, there was no significant difference in the total number of MCs detected with toluidine blue and the number of IgE+ cells, implying that the majority of IgE+ cells were mast cells (data not shown).

In non-sensitized dogs, the number of toluidine blue stained MCs and the number of double labelled MCs did not differ significantly between dogs, which were exposed to fleas and control dogs. In contrast, biopsies from flea-sensitized dogs had significantly higher numbers ($P < 0.001$) of double-labelled MCs than toluidine blue stained MCs. These findings were present independent of flea exposure. The data are shown in Fig. 2.

In all groups and with all staining techniques used, results were comparable at the site of flea exposure and at the control site.

Three MC subtypes were identified in the skin of flea-sensitized and non-sensitized dogs. T-MCs, which stain red with an anti-tryptase antibody, C-MCs that stain blue using an enzyme-histochemical detection for chymase, and TC-MCs, which contain

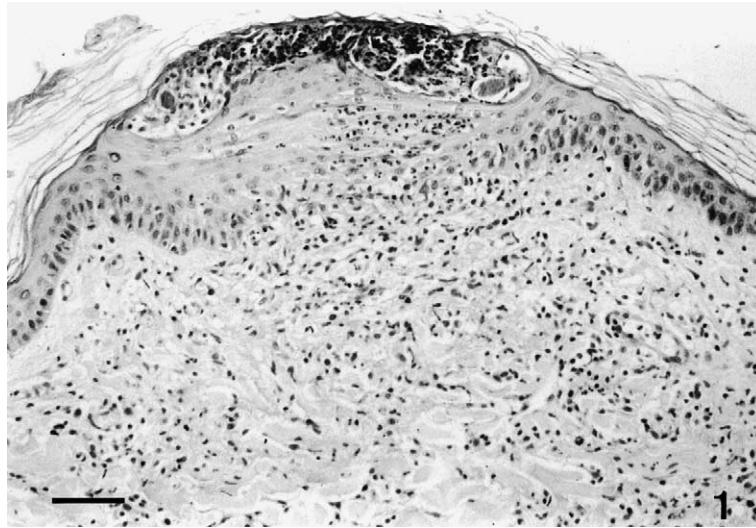


Fig. 1. Skin biopsy of a flea-sensitized dog 1 day after flea exposure. Note the intraepidermal eosinophilic pustule and the perivascular dermal infiltration. H&E, bar = 100 μ m.

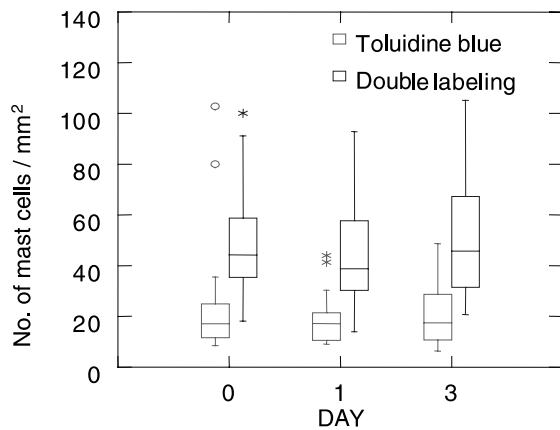


Fig. 2. Mast cell density in flea-sensitized dogs without flea exposure. Note the significantly higher number of double-labelled mast cells compared to toluidine blue stained mast cells ($P < 0.001$). The data are presented in box plots. 80% of the data are within the box. The bar within the box represents the mean value. The symbols outside the box represent far values.

both tryptase and chymase, and thus stain red and blue.

Examination of the percentage distribution of the three mast cell subtypes (C-, TC-, and T-MC) and comparing flea-sensitized dogs before and after flea exposure showed statistically significant changes. The percentage of C-MC increased significantly at day 1 ($P < 0.001$) and day 3 ($P < 0.001$) compared with day 0 at the site of flea exposure. Similar results were found at the site of flea exposure and at

the control site ($P < 0.001$ day 0 *versus* day 1; $P < 0.05$ day 0 *versus* day 3) (Fig. 3). In contrast, the percentage of TC-MC decreased significantly at day 1 ($P < 0.001$) and day 3 ($P < 0.05$) compared to day 0 at the site of flea bites. Once again, the results at the control site were similar, the percentage of TC-

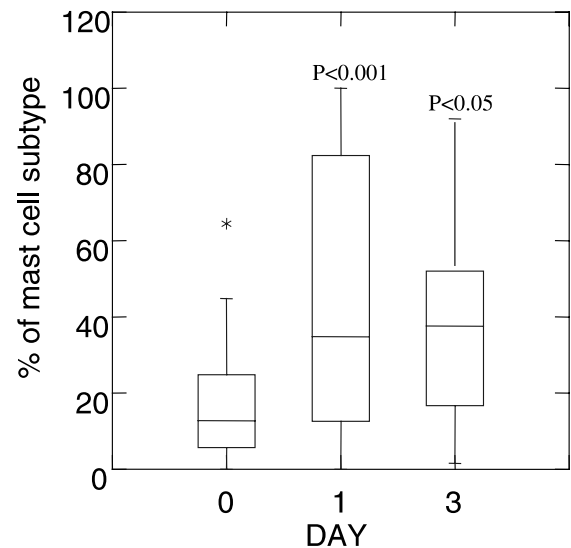


Fig. 3. Percentage of C-mast cells in flea-sensitized dogs after flea exposure at the control site. Note the significant increase of this mast cell subtype at days 1 and 3 compared to day 0 (P values: day 0 *versus* day 1 or 3, respectively). The data are presented in box plots. 80% of the data are within the box. The bar within the box represents the mean value. The symbols outside the box represent far values.

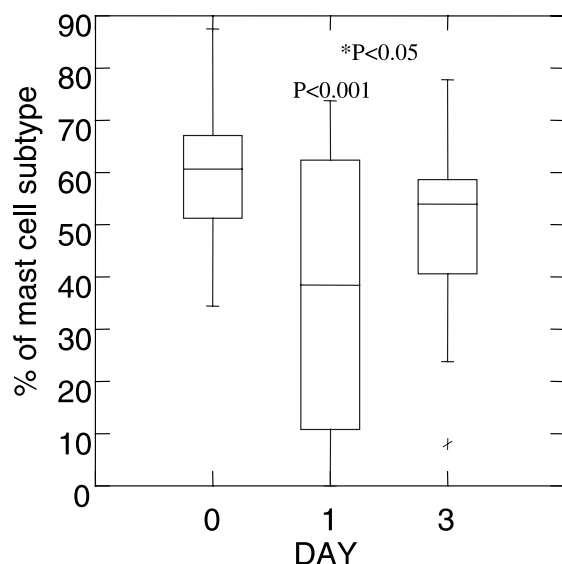


Fig. 4. Percentage of TC-mast cells in flea-sensitized dogs after flea exposure at the site of flea exposure. Note the significant decrease of this mast cell subtype at days 1 and 3 compared to day 0 (P values: day 0 *versus* day 1 or 3, respectively). The data are presented in box plots; 80% of the data are within the box. The bar within the box represents the mean value. The symbols outside the box represent far values.

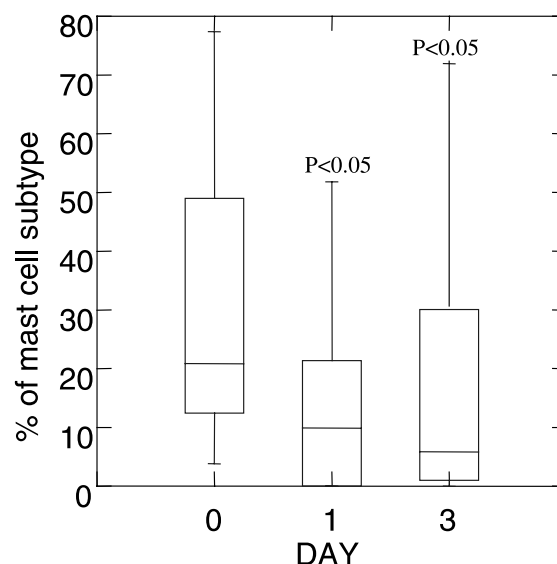


Fig. 5. Percentage of T-Mast cells in flea-sensitized dogs after flea feeding at the site of flea exposure. Note the significant decrease of this mast cell subtype at days 1 and 3 compared to day 0 (P values: day 0 *versus* day 1 or 3, respectively). The data are presented in box plots. 80% of the data are within the Box. The bar within the box represents the mean value. The symbols outside the box represent far values.

MC decreased ($P < 0.05$ day 0 *versus* day 1) (Fig. 4). For this mast cell subtype, comparison of day 1 and day 3 showed an increase at day 3 at both the sites ($P < 0.05$ day 1 *versus* day 3). The percentage of T-MC similarly decreased ($P < 0.05$ day 0 *versus* day 1; $P < 0.05$ day 0 *versus* day 3). For this subtype no rebound was seen comparing day 1 with day 3 (Fig. 5). A similar trend, although statistically not significant, was found at the control site, possibly due to the low absolute numbers of this MC subtype.

Skin tests were positive in all flea-sensitized dogs after 20 min. The majority of dogs showed signs of delayed type hypersensitivity after 24 and 48 h. Skin tests were negative in the non-sensitized dogs (data not shown).

High serum titres of flea-specific serum IgE and IgG antibodies were detectable in all flea-sensitized dogs. The non-sensitized dogs had negative serum titres (data not shown).

DISCUSSION

The study was carried out to investigate the effect of sensitization to flea antigen and flea exposure on skin histopathology, MC density, the percentage distribution of MC subtypes and IgE+ cells.

Histopathological findings in biopsy specimens from the sites of flea bites in both, the flea-sensitized and the non-sensitized dogs, are in agreement with the findings of Gross and Halliwell (1985), who found a predominantly eosinophilic infiltrate at the site of flea bites or after intradermal skin test for up to 24 h after which time the infiltrates were mainly mononuclear. In our biopsies at day 1 after flea exposure, the histopathological changes at the site of flea exposure were characterized by perivascular infiltration with MCs and eosinophils mixed with lymphocytes, histiocytes, and some neutrophils. At day 3 after flea exposure, the perivascular infiltrate was composed mainly of mononuclear cells and of MCs. Therefore our findings confirm that features of both, immediate (IgE-mediated) and delayed (cell-mediated) hypersensitivity reactions, are evident.

By toluidine blue staining, no statistically significant differences were detectable in MC density in the dermis of the four groups of dogs independent of the day biopsies were taken. To our knowledge MC density has never been investigated quantitatively in dogs with FBH. Another study on FBH found small to moderate mast cell numbers around blood vessels in the majority of dogs at all stages of

the response to flea antigen, except at 15 min post-exposure (Gross & Halliwell, 1985). Since this study was based on semiquantitative observations, the results are not comparable with ours. In a study of MC density in the skin of dogs with atopic dermatitis, approximately the same MC density was detected in the skin of atopic and control dogs (Welle *et al.*, 1999b). This finding is supported by a study of atopic dermatitis in humans, where MC numbers are normal if detected by metachromatic staining procedures (Soter, 1989).

Similar results were obtained with the staining for IgE+ cells. No significant changes in the density of IgE+ cells were found between non-sensitized dogs and flea-sensitized dogs. To our knowledge no study has been performed to detect IgE+ cells in dogs with FBH. It is known that mast cells and basophils express large numbers of FcεRI on their cell surface; however, under some circumstances, notably in human, other effector cells express at least small numbers of this receptor. These cells include Langerhans cells, blood monocytes and macrophages, circulating dendritic cells, and eosinophils (Turner & Kinet, 1999). It has been shown that epidermal (i.e., Langerhans cells) and dermal dendritic cells in the skin of dogs with atopic dermatitis express surface IgE. In contrast, IgE expression was not shown in the skin of control dogs (Olivry *et al.*, 1996). Other authors have shown an increased number of IgE+ cells in the skin of horses with insect bite hypersensitivity (Van der Haegen *et al.*, 2001). These reports suggest that the increased number of IgE+ cells is a result of epidermal and dermal dendritic cell proliferation. These two cell types appear to be crucial for IgE-mediated allergen capture and presentation in human atopic dermatitis. Our results may indicate that dendritic cells are not involved in allergen uptake in FBH. Possibly the epicutaneously applied allergen in FBH is taken up by other antigen presenting cells such as keratinocytes, dermal mast cells, and endothelial cells.

Three MC subtypes were identified in the skin of flea-sensitized dogs and non-sensitized dogs, in agreement with previous findings (Kube *et al.*, 1998).

In non-sensitized dogs, the number of toluidine blue stained MCs and the number of double-labelled MCs did not differ significantly in dog skin whether or not they were exposed to fleas. In contrast, significantly higher numbers of double labelled MCs compared with toluidine blue stained MCs were detectable in flea-sensitized dogs, inde-

pendent of flea exposure. The toluidine blue staining of mast cells depends on the affinity of metachromatic dyes for the cytoplasmic granules, whereas double labelling selectively stains the mast cell proteases. Therefore the apparent increase in MC numbers after double labelling in flea-sensitized dogs is most likely a result of an upregulation of MC proteases in response to sensitization. The lower number of MCs staining with toluidine blue could reflect degranulation of acid glycosaminoglycans. A gradual mediator release from MCs has been shown in certain chronic disease states (e.g., bullous pemphigoid), indicating that different degranulation patterns may reflect granule heterogeneity with varying responses to different IgE-mediated stimuli (Kaminer *et al.*, 1995).

Investigating the percentage distribution of MCs subtypes, we did not find any differences between flea-sensitized and non-sensitized dogs before exposure to fleas. After flea exposure, the percentage of C-MC went up at days 1 and 3 compared to day 0 and the percentages of TC-, and T-MC went down during the same time period. These results provide strong evidence that a selective degranulation of MC tryptase occurs in response to flea feeding. Thus, former TC-MCs change to the C-MC subtype, which results in a higher percentage of C-MCs and a lower percentage of TC-MC. The increase of tryptase positive MCs and the decrease of chymase positive MCs from day 1 to 3 suggest regranulation with tryptase. These data suggest that tryptase may play a role in the reaction to flea exposure in dogs with FBH. Tryptase has been implicated as an important mediator in the pathology of numerous allergic and inflammatory conditions in human such as rhinitis, conjunctivitis, and asthma (Rice *et al.*, 1998). In addition, our results indicate that granule heterogeneity exists in the dog and suggest that selective degranulation of tryptase occurs depending on the pathological condition affecting skin of the dog. A selective degranulation of tryptase has already been proposed in dogs with cutaneous mucinosis (Welle *et al.*, 1999a). However, double-immunolabelling experiments in human MCs demonstrated that chymase and tryptase are stored in the same secretory granules (Craig *et al.*, 1988). According to our findings in dogs, either this is not the case and tryptase and chymase are stored in separate granules, or, if they are stored in the same granules, tryptase can be released independently of chymase. Extrapolation of this question would require detailed double-immunoelectronmicroscopy in dog skin MCs.

With regard to MC density, subtypes, and density of IgE+ cells, we obtained comparable results at the site of flea exposure and the control site in all groups. These findings indicate a systemic reaction to the sensitization and to the challenge with the antigen.

Our data underline the importance of further studies on the *in vitro* and *in vivo* properties of mast cell proteases and subtypes. The first step will be to quantify protease release and measure the upregulation of the mRNA encoding tryptase and chymase. These results could further elucidate the pathophysiological role of these MC proteases in hypersensitivity reactions.

High serum titres of flea-specific serum IgE and IgG antibodies were detectable in all flea-sensitized dogs. The non-sensitized dogs had negative serum titres. This finding is supported by a study in dogs and cats with confirmed allergic dermatitis to flea antigens, where the authors found significantly more specific IgE and IgG as compared to the controls (Halliwell & Longino, 1985; Foster *et al.*, 1997).

With regard to skin tests, both immediate- and delayed-type hypersensitivity reactions developed in a random sequence with most dogs showing both types of reactions during the course of the study. This finding is in agreement with the results of Gross and Halliwell (1985). Both reaction types have as well been described in human (Goldman *et al.*, 1952; Feingold & Benjamini, 1961), guinea pigs (Benjamini *et al.*, 1961; Larrivee *et al.*, 1964) and cats (Foster *et al.*, 1997).

Our data indicate that the immunopathogenesis of flea allergic dermatitis is highly complex, involving an immediate and a delayed type of allergic reaction and involving not only histamine release and T-cell responses, but also upregulation of mast cell proteases and their selective release. Since mast cell subtypes in dog and human can be distinguished according to their content of the neutral proteases chymase and tryptase, investigations of the involvement of these proteases in allergic disorders in sensitized dogs could serve as a good model for allergic disease in man (Welle, 1997).

ACKNOWLEDGMENTS

We thank Ilkka Harvima, Department of Dermatology, University of Kuopio, Finland for providing us with the anti-tryptase antibody, Ursula Forster, Catherine Rudaz, Manuela Bozzo, Evelyn Rohrer, and Erika Garchi for their excellent technical assistance and the animal caretakers of the Novartis dog facilities in St. Aubin.

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(Accepted for publication 8 May 2002)