



Immune dysregulation in flea allergy dermatitis—A model for the immunopathogenesis of allergic dermatitis

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Abstract

Background: Flea allergy dermatitis (FAD) is a common skin disease in dogs and can be induced experimentally. It often coexists with other allergic conditions. So far no studies have investigated the quantitative production of cytokine mRNA in skin biopsies and peripheral blood mononuclear cells (PBMC) in flea allergic dogs.

Objective: The aim of our study was to improve the understanding of the immunopathogenesis of allergic dermatitis as a response to fleabites.

Material and methods: Allergic and non-allergic dogs were exposed to fleas. Before and after 4 days of flea exposure mRNA was isolated from biopsies and PBMC. Production of chymase, tryptase, IL-4, IL-5, IL-13, TNF- α and IFN- γ mRNA was measured by real-time RT-PCR. The inflammatory infiltrate in the skin was scored semi-quantitatively. The number of eosinophils, mast cells (MC) and IgE+ cells/mm² was evaluated to complete the picture.

Results: FAD was associated with a higher number of MC before flea exposure and with a significant increase of eosinophils after flea exposure as compared to non-allergic dogs. The number of IgE+ cells was higher in allergic dogs before and after flea exposure. In allergic dogs mRNA for most cytokines and proteases tested was higher before flea exposure than after flea exposure. After exposure to fleas an increased mRNA production was only observed in non-allergic dogs. In vitro stimulation with flea antigen resulted in a decreased expression of most cytokines in allergic dogs before flea exposure. In contrast, in PBMC, only increased levels of IL-4 and IL-5 mRNA were observed in allergic dogs before flea exposure. However, after flea exposure and additional stimulation with flea antigen the production of mRNA for all cytokines tested was significantly increased in allergic dogs.

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Conclusion: We demonstrated that the response in biopsies and PBMC is different and that FAD is associated with a TH2 response.

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1. Introduction

Allergic disease is a complex phenomenon that involves both, inflammatory cells and structural cells, such as fibroblasts and endothelium. Cytokines produced in response to specific antigens/allergens are important in controlling the function of the inflammatory cells and the isotype of antibodies produced by B-cells (Finkelman et al., 1990; Paul and Seder, 1994). Therefore, these cytokines are likely to be important in the pathogenesis of allergic disease. A number of studies have suggested that T-cells producing TH2 cytokines are responsible for the induction and maintenance of the allergic response (Romagnani, 1994). Furthermore, it is well known that MC and eosinophils are important key players in allergic disease. MC are stimulated via the immunoglobulin E (IgE) receptor (Fc epsilon RI) to release proinflammatory mediators such as TNF- α , reactive oxygen species, such as nitric oxide, proteases and lipid-derived mediators. Furthermore, MC specific proteases, particularly tryptase and chymase, are involved in the pathogenesis of allergic diseases in man (Harvima et al., 1990, 1988) and in dogs (Welle et al., 1999a,b). The most important effector function of eosinophils in allergic disease is the release of reactive oxygen species and toxic eosinophilic granule proteins that result in local propagation of inflammation and tissue damage (Elsner and Kapp, 1999; Rothenberg, 1998). In addition, eosinophils produce cytokines that are involved in the regulation of allergic inflammation (Kay et al., 1997; Rothenberg, 1998).

FAD, usually caused by infestation with *Ctenocephalides felis felis*, is one of the more common allergic disease 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 (AD) (Kwochka, 1987; Reedy and Miller, 1989). Typical signs of FAD are pruritus and

popular dermatitis which occur most frequently in the lumbosacral area, around the anus, the caudo-medial thighs, the ventral abdomen and the flanks (Nesbitt and Schmitz, 1978). Why allergic disease develops only in some animals, although many dogs are equally exposed to the antigens, is not completely understood (Bonay et al., 1997).

Until now, research in canine allergic disease has been restricted to analyses of skin biopsies, circulating IgE levels and the local reaction to intradermally applied antigens. Few studies have investigated the involvement of cytokines in allergic skin disease (Hayashiya et al., 2002; Nuttall et al., 2002a). The influence of IgE levels on Fc epsilon RI expression and its effect on TNF- α production from canine skin MC has been demonstrated (Brazis et al., 2002). In a study using semi-quantitative RT-PCR to determine the production of gene transcripts for immunosuppressive cytokines, as well as TH1 and TH2 cytokines in skin biopsies from dogs with AD investigators reported overexpression of IL-4 mRNA and a reduced transcription of TGF- β compared to healthy skin. Furthermore higher levels of IFN- γ , TNF- α and IL-2 mRNA were seen in lesional compared to non-lesional skin of atopic dogs (Nuttall et al., 2002a). In an other study the presence of TH2 cytokines (IL-4 and IL-5) is reported in acute lesions of AD whereas in chronic lesions IFN- γ is found (Sinke et al., 2002). Investigations concerning cytokine expression by PBMC in response to allergens are very limited and have so far only been done in canine AD (Nuttall et al., 2002b). Currently no information is available concerning the profile of cytokines produced by PBMC and by immunoregulatory cells in the skin in response to flea antigen.

The aim of our study was to improve the understanding of the immunopathogenesis of the allergic response by inducing a local allergic reaction in dogs by standardized local flea exposure. Therefore, we investigated the production of IL-4, IL-5, IL-13,

TNF- α and IFN- γ mRNA by real-time RT-PCR in skin biopsies and PBMC and, in addition, we looked at chymase and tryptase in skin biopsies. Production of mRNA was quantified in allergic and non-allergic dogs after *in vitro* stimulation with flea antigen before and after the *in vivo* exposure to fleas. Furthermore, we investigated the clinical response, and scored the inflammatory infiltrate in the skin semi-quantitatively. The number of eosinophils, MC and IgE+ cells/mm² was evaluated to complete the picture.

In human beings it is difficult to study the development of the allergic response to antigens, since it is not possible to expose allergic and non-allergic individuals in a standardized manner. Most of the studies investigating cytokine expression in allergic individuals have been performed by stimulating PBMC since skin biopsies are not easily available. The dog is one of the few species other than humans in which allergy develops naturally on normal environmental exposure to a broad spectrum of allergens including pollens, house dust mites, human dander, fleas and food (Mueller et al., 2000; Paterson, 1995; Sture et al., 1995). Since dogs have a comparable clinical response to important human allergens, they can be regarded as a model to study the pathogenesis of allergies. In our dog model, FAD can be induced under experimental conditions. Therefore, the allergic dermatitis in these animals is a suitable model for papular urticaria, a histological similar response in humans resulting from a hypersensitivity reaction to arthropod bites as well as other allergic skin disorders such as AD in humans.

2. Material and methods

2.1. Animals

Twenty beagles from the research dog colony at the Novartis Centre de Recherche Santé Animale in St.-Aubin, Switzerland were used for this experiment. These dogs had been used before as control group in an experiment where antiparasitic compounds were tested. During this trial they have had continuous full body flea exposure for a maximum of 6 weeks. During this experiment a number of dogs developed signs of FAD starting with pruritus, wheals, erythema and papules mainly in the dorsal lumbosacral area, the

caudomedial thighs and the flanks. In some dogs lesions progressed and led to alopecia and severe crusting in the above described locations. At this point dogs were withdrawn from the experiment. Other dogs used in the same experimental setting never showed any clinical signs. Ten of the dogs with clinical FAD and 10 dogs that had never shown any signs of FAD were recruited for this experiment. Before the start of this experiment the dogs had not been exposed to fleas for at least 2 months. The studies have been approved 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).

2.2. Flea exposure

All dogs were exposed to fleas in the manner described here. For 4 days (days 1–4) small cages (round, 3.5 cm in diameter, covered with a fine mesh membrane) containing 30 live fleas were attached with an elastic bandage to an area on the lateral thorax that had previously been shaved. The cages were left in place for 30 min to allow the fleas to feed through the membrane. After feeding the fleas were left in the cages and were frozen at -18°C . This enabled us to check by microscopic evaluation how many fleas were engorged. Throughout the experiment the percentage of engorged fleas was between 56 and 100% (data not shown).

2.3. Intradermal test

IDTs were performed in all dogs on the first day before flea exposure and on day 4 of the experiment to confirm the presence or absence of a hypersensitivity reaction. The reagents used were whole flea extract (Greer Laboratories, Lenoir, NC, USA) at a test concentration of 0.6 $\mu\text{g/ml}$. Histamine (Center Laboratories, Port Washington, NY, USA) was used as positive control at a concentration of 10 $\mu\text{g/ml}$ and physiologic saline solution (0.9%) served as negative control. We injected 100 μl of each test solution intradermally on the shaved abdomen of the dogs. The diameter and the thickness of the wheal and flare reaction at the sites of flea antigen injection were measured with a sliding caliper 20 min after the intradermal injection. The final IDT score (ranging from 0 to 3) was consisting of the mean of two scores

which were obtained by measuring of the diameter and the thickness of the wheals. The score for the diameter was obtained as follows: if the diameter was at least 1 mm smaller than the histamine reaction the score was 1; if the diameter was equal to the histamine reaction (± 1 mm) the score was 2 and if the diameter was at least 1 mm greater than the histamine reaction the score was 3. The score for the thickness was obtained as follows: if the diameter was at least 0.5 mm smaller than the histamine reaction the score was 1; if the diameter was equal to the histamine reaction (± 0.5 mm) the score was 2 and if the diameter was at least 0.5 mm greater than the histamine reaction the score was 3. Final scores greater than 1 were considered to be positive.

2.4. Skin biopsies

Two 8 mm punch biopsies were taken, under local anesthesia (Lidocain 2%, 1.5 ml; Chassot AG, Belp, Switzerland), from each dog on day 1 before flea exposure. Two further biopsies were then taken from each dog on day 4 before flea exposure. At each time point half of one biopsy was fixed in formalin (4%) at room temperature for 24 h. The samples were then embedded in paraffin wax and serial sections of 4 μ m were cut from each tissue block.

The rest of the material was incubated in Ringer's solution or stimulated as described later.

One section of each paraffin-embedded 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. Eosinophilic granulocytes were demonstrated with the Luna's method (Luna, 1968). IgE+ cells were stained immunohistochemically with a canine IgE-specific antibody raised in chicken (Griot-Wenk et al., 1998) as described previously (Egli et al., 2002).

In order to confirm our quantitative RT-PCR results, which we obtained from the Ringer incubated biopsies at day 1 mRNA was extracted from the remaining paraffin-embedded tissue of the day 1 biopsies.

Histopathology of the H&E slides was interpreted using a semi-quantitative grading system (0, 1, 2, 3) to assess the degree (none, mild, moderate, severe) of cellular infiltration (e.g. lymphocytes, mononuclear cells, eosinophils, MCs) in the dermis. The changes in the epidermis (increased epidermal thickness, spon-

giosis and cellular infiltration) were similarly graded. The final score for each biopsy was the mean of the grade attributed to the dermis and that to the epidermis. All slides were analysed by the same person, who was blind with regard to the group and day assignment of biopsies.

The quantification of positively stained MC, IgE+ cells, and eosinophils was performed by evaluation of the total area of each section using a square eyepiece graticule (objective $\times 25$, eyepiece $\times 10$, 10×10 squares with a total area of 0.0156 mm^2). All positively stained cells within the dermis with a visible nucleus were counted. Cell numbers are expressed as cells/ mm^2 .

The remaining 11/2 biopsies were divided into three equal parts and were incubated in 500 μ l Ringer's solution (diluent), in diluent containing compound 48/80 (5 μ g/ml), or whole flea antigen (0.6 μ g/ml) (Greer Laboratories, Lenoir). Compound 48/80 was chosen as a non-immunological stimulus and whole flea antigen was chosen as an IgE-dependent stimulus. After 6 h of incubation at 37 $^{\circ}\text{C}$, any reaction was stopped by sonication and the biopsies were put in 1 ml Trizol reagent (Invitrogen AG, Basel, Switzerland) and stored at -20°C until mRNA was extracted.

2.5. Blood sample collection, PBMC separation and cell culture

Before the first flea exposure and at day 4 of flea exposure whole blood (30 ml) was collected into EDTA-coated tubes (S-Monovette, Sarsedt, Sevelen, Switzerland) by jugular venepuncture. To obtain PBMC whole blood was diluted 1:1 in chilled PBS/1.2% tri-sodium citrate and layered over a 1.077g/ml polysucrose/sodium diatrizoate solution (Ficoll-Paques Plus, Amerhsam Biosciences, Otelfingen, Switzerland). The samples were centrifuged at $400 \times g$ (1800 rpm) for 30 min at 20 $^{\circ}\text{C}$. Cells were collected from the interface and washed three times in chilled citrated PBS. The cell pellet was re-suspended in 2 ml of Dulbecco's modified Eagle's medium (Sigma-Aldrich, Fluka Chemie GmbH, Buchs, Switzerland), supplemented with 10% FCS (hereafter referred to as complete medium) and counted. Viability was determined by trypan blue dye exclusion. The cells were plated in a final concentration of 2×10^6 cells/well in 24 well culture plates (Techno Plastic Products, Trasadingen, Switzerland) and

cultured at 38 °C in 5% CO₂ for 24 h. Cells were incubated in complete medium (1 ml), which was supplemented either with 0.6 µg/well of whole flea antigen (Greer Laboratories, Lenoir), 5 µg/well of ConA (positive control), or Ringer's solution (negative control). For each dog and each stimulus a minimum of 3 wells was plated. The supernatants from the culture plates were harvested into Eppendorf tubes and centrifuged for 10 min at 200 × *g* (1200 rpm). The supernatants were saved in new Eppendorf tubes and frozen at –80 °C to await analysis. The cell pellet from the Eppendorf tube was resuspended with 1 ml Trizol reagent (Invitrogen AG) and pooled with the cells remaining in the culture wells. Cells were stored at –20 °C until mRNA was extracted.

2.6. mRNA extraction and quantitative real-time RT-PCR

Real-time RT-PCR was performed to quantify the production of mRNA of the MC proteases tryptase and chymase, canine (c)IL-4, IL-5, IL-13, IFN-γ, TNF-α and ribosomal (r)18S in the skin biopsies. Before RNA extraction, biopsies were homogenized in Trizol reagent (Invitrogen AG) with an Ultra-Turrax. In PBMC the expression of mRNA for cIL-4, IL-5, IL-13, TNF-α, IFN-γ and r18S was investigated. Total RNA was extracted from Trizol according to the manufacturer's instructions with the following modification. We used phenol:chloroform:alcohol in the proportion 25:24:1 for the second extraction. After extraction, RNA was dissolved in 20 µl of RNase-free water and the concentration of purified total RNA was determined by measuring the optical density at 260 nm. DNase (Promega, Catalys AG, Wallisellen, Switzerland) treatment followed according to manufacturer's protocol with extension of the incubation in the stop solution for 30 min. Two microgram of each RNA sample were reverse transcribed into cDNA using random hexamer primers (Promega, Catalys AG) in a final reaction volume of 50 µl. Primers and fluorescent FAM-labeled probes were designed in-house from published sequences using the PrimerExpress software (Applied Biosystems, Foster City, USA). The sequences of the primers and probes are shown in Table 1. Real-time RT-PCR was performed using the TaqMan Universal PCR Master Mix (Invitrogen AG) with a concentration of primers and probes of 1000 and 200 nM, respectively,

in a final reaction volume of 25 µl in an ABI PRISM 7700 Sequence Detector (Applied Biosystems) according to the manufacturer's protocol. The probe for chymase was used at a concentration of 50 nM. PCR amplification was performed for 40 cycles. Control experiments using RNA samples without reverse transcription were performed to demonstrate absence of possible contaminating genomic DNA. PCR results from either biopsies or PBMC of non-allergic dogs before flea exposure and subsequent *in vitro* treatment with Ringer were used as a reference value and defined to be 1. PCR amplification of the constitutively expressed r18S gene was used as a measure of input RNA and was used to normalize our mRNA levels. These normalized values were then measured relative to our reference value. The final results of the cytokine and protease mRNA production are expressed as relative units, which were calculated according to the manufacturer's protocol (User Bulletin #2, Applied Biosystems).

2.7. Statistics

Basic descriptive statistics and Box-and-Whisker plots were used to describe and visualize the results. The differences with regard to the inflammation score, the number of MC, IgE+ cells and eosinophils and the cytokine mRNA production in biopsies between days 1 and 4 within the allergic and the non-allergic group were evaluated with the paired Wilcoxon Signed-Rank Test for difference in medians. This test was also applied to assess the difference in mRNA cytokine production in PBMC within each group comparing day 1 before flea exposure and day 4. For assessment of the differences in all relevant parameters between the groups (allergic dogs versus non-allergic dogs) analysis using the non-parametric Kruskal–Wallis One-Way ANOVA on Ranks test was used. The Bonferroni correction was used to correct for multiple comparisons. In the samples incubated in Ringer's solution on day 1, the differences were calculated with the Wilcoxon Signed-Rank Test (One-Sample) because the non-allergic dogs were defined to be 1 (reference value). The overall *p*-value for statistical significance was set to 0.05. Statistical analysis was performed with the software package NCSS 2001 (www.ncss.com).

Table 1

Sequences of primers and probes used for quantification of mRNA production by real-time RT-PCR

Cytokine	Gene [GeneBank accession number]	Sequences of primers and probes
Chymase	J02904	5'-CAGCTGTGTGTTGGCAATCC-3' (sense) 5'-GGACACAATTCCTGGGCTA-3' (antisense) 5'-CCCCTGAATCTCCCTTAAATGCAGAT-3' (probe) ^a
Tryptase	M24664	5'-CACTGTGTGGGACCGAACG-3' (sense) 5'-CCCCATTCTCGGGTGTGTAG-3' (antisense) 5'-CTGAACCCTGATTTCCTCAGGACACACG-3' (probe) ^a
IL-4	AF187322	5'-AACGACTCGTGCATGGAGCT-3' (sense) 5'-TGCCGCAGTAGCAGTAGCAGC-3' (antisense) 5'-CACTGCTCCAAAGAACACAAGCGATAAG-3' (probe) ^a
IL-5	AF091133	5'-TGGTGGCAGAGACCTTGACA-3' (sense) 5'-CATCAGGTTCCTCATCGCCTA-3' (antisense) 5'-TGCTCTCCACTCATCGAACTTGGCTG-3' (probe)
IL-13	AF244915	5'-TGGTGTGGAGCGTCAACCT-3' (sense) 5'-CGGAGACATTGATCAGAGATTCTAGA-3' (antisense) 5'-ACCGCCGGCATGTACTGCGC-3' (probe)
TNF- α	S74068	5'-CCCCAAGTGACAAGCCAGTAG-3' (sense) 5'-AGCCACTGGAGCTGCCC-3' (antisense) 5'-TCATGTTGTAGCAAACCCGAAGCTGA-3' (probe)
IFN- γ	AF126247	5'-CAGCACCAGTAAGAGGGAGGA-3' (sense) 5'-TCATTTATCGCCTTGCGCT-3' (antisense) 5'-CTTAAGCTGATTCAAATTCCTGTGAACGAT-3' (probe)

Sequences of primers and probes for the canine cytokines and proteases were designed in-house from published sequences using the PrimerExpress software (Applied Biosystems, Rotkreuz, Switzerland). Primers and probe for the housekeeping gene r18S (ribosomal RNA) were purchased as predeveloped Control Reagents (Applera Europe B.V., Rotkreuz, Switzerland).

^a Probes span exon–exon junction.

3. Results

3.1. Intradermal test

Skin test results demonstrated that allergic dogs had a much stronger response compared to non-allergic dogs, both before flea exposure (day 1) and at day 4. When inoculated with whole flea antigen (Greer Laboratories, Lenoir), allergic dogs had an average score of 2 at day 1 and 2.1 at day 4, whereas non-allergic dogs had an average score of 0.1 at both days (data not shown).

3.2. Inflammation score

The inflammation score in biopsies from allergic dogs was significantly higher than in biopsies from non-allergic dogs, both at day 1 before flea exposure and at day 4 ($p < 0.05$; Fig. 1a and b).

Exposure to fleas resulted in a significant aggravation of the inflammatory response in both groups

($p < 0.05$). In allergic dogs the mean score increased from 1 to 2 after flea exposure and in non-allergic dogs it increased from 0.3 to 1.2 (Fig. 1a and b).

3.3. Mast cell density

Before flea exposure, the number of MC was significantly higher in the allergic group compared to the non-allergic group ($p < 0.05$; Fig. 2a). In response to flea exposure MC density decreased significantly in the allergic dogs, whereas in non-allergic dogs MC numbers did not change (Fig. 2a and b). Therefore, on day 4 no significant difference between the two groups (Fig. 2b) could be observed.

3.4. IgE+ cell density

The number of IgE+ cells in biopsies from allergic dogs was significantly higher than in biopsies from

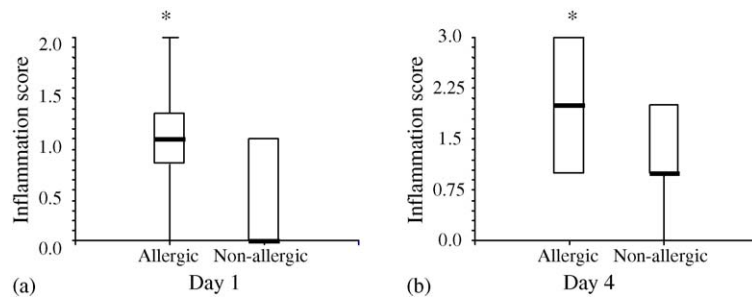


Fig. 1. Inflammation score at day 1 and day 4 of allergic and non-allergic dogs. Note that scores in allergic dogs are significantly higher than in non-allergic dogs, both at day 1 and day 4 (a and b) ($p < 0.05$). Values in the boxes reflect the 25th, 50th (median) and 75th percentiles. The whiskers represent approximately 90–95% of the values.

non-allergic dogs, both at day 1 before flea exposure and at day 4 ($p < 0.05$; Fig. 3a and b).

3.5. Eosinophil density

Before flea exposure the number of eosinophils was very low in both groups (Fig. 4a) and no significant difference between the two groups could be observed. In response to flea exposure both groups react with an increase in eosinophil density but this increase was much more severe in the allergic group ($p < 0.05$; Fig. 4a and b).

3.6. Production of cytokine and protease mRNA in skin biopsies

The results of the cytokine and protease mRNA expression in skin biopsies from non-allergic and allergic dogs are summarized in Tables 2 and 3. In the

following, we will comment on the most important results.

A. Biopsies incubated with Ringer's solution:

- On day 1, a significantly higher amount of mRNA of all cytokines and proteases tested was measured in biopsies from allergic dogs (except IL-4) (Table 3). These results were confirmed by investigating the mRNA production in the paraffin-embedded biopsies, which were taken on day 1 before flea exposure and immediately fixed in formalin (data not shown).
- Exposure to fleas for 4 days resulted in a higher mRNA production of cytokines (except IFN- α) and proteases in non-allergic dogs. In contrast, in the allergic group, flea exposure did not result in a significant increase of cytokine and protease mRNA production (Table 2).
- After flea exposure, no significant difference in cytokine and protease mRNA production

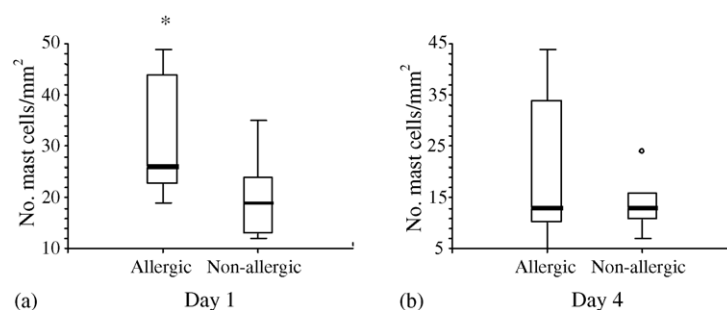


Fig. 2. Mast cell density at day 1 and day 4 comparing allergic and non-allergic dogs. Note that at day 1 mast cell numbers in allergic dogs are significantly higher than in non-allergic dogs (a) ($p < 0.05$). No difference can be observed at day 4 (b). Values in the box plots reflect the 25th, 50th (median) and 75th percentiles. The whiskers represent approximately 90–95% of the values. Moderate (○) outliers are represented by dots.

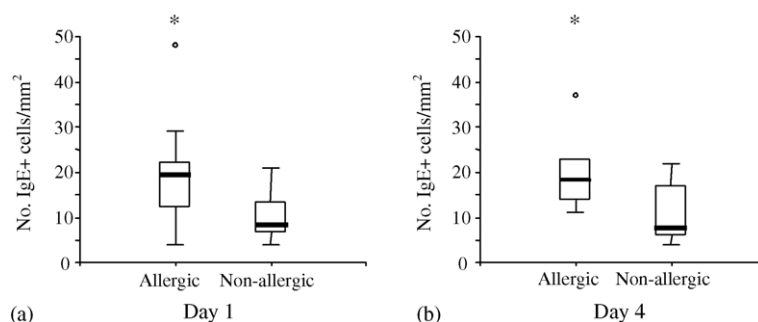


Fig. 3. Density of IgE+ cells at day 1 and day 4 comparing allergic and non-allergic dogs. Note that density in allergic dogs is significantly higher than in non-allergic dogs, both at day 1 and day 4 (a and b) ($p < 0.05$). Values in the box reflect the 25th, 50th (median) and 75th percentiles. The whiskers represent approximately 90–95% of the values. Moderate (○) outliers are represented by dots.

between non-allergic and allergic dogs was noted, although a strong difference with regard to the median values was observed (Table 3).

B. Biopsies stimulated with whole flea antigen (Greer):

- Before flea exposure allergic dogs showed a lower production of chymase, IL-4, IL-5, TNF- α and IFN- γ mRNA compared to non-allergic dogs (Table 3).
- After flea exposure, the production of tryptase and TNF- α mRNA was lower in the allergic group, whereas the mRNA production of chymase and the other cytokines was comparable between the two groups (Table 3).

3.7. Production of cytokine mRNA in PBMC

The results of the cytokine expression in PBMC from non-allergic and allergic dogs are summarized in

Tables 4 and 5. In the following, we will comment on the most striking results.

A. PBMC stimulated with Ringer's solution:

- Before flea exposure allergic dogs had a significantly higher mRNA expression of IL-4 and IL-5 than non-allergic dogs (Table 5).
- After flea exposure the significantly higher amount of IL-4 and IL-5 mRNA remained. The expression of mRNA for IFN- γ and TNF- α was significantly lower than in non-allergic dogs (Table 5).

B. PBMC stimulated with whole flea antigen:

- Before flea exposure allergic dogs had a significantly higher mRNA expression of IL-4 and TNF- α than non-allergic dogs (Table 5).
- After flea exposure a significantly higher mRNA amount of all cytokines was measured in

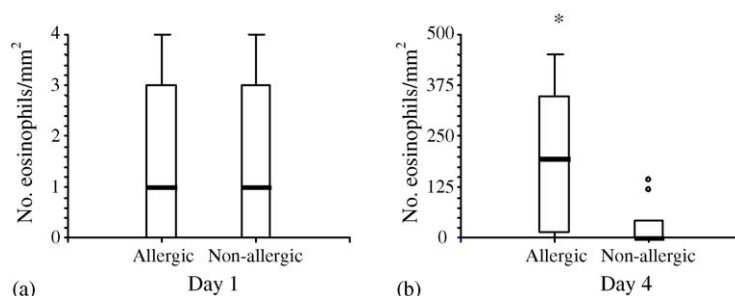


Fig. 4. Eosinophil density at day 1 and day 4 comparing allergic and non-allergic dogs. No difference can be observed at day 1 (a), whereas at day 4 (b) the number of eosinophils in allergic dogs is significantly higher than in non-allergic dogs ($p < 0.05$). Values in the box reflect the 25th, 50th (median) and 75th percentiles. The whiskers represent approximately 90–95% of the values. Moderate (○) outliers are represented by dots.

Table 2

Changes in mRNA production in skin biopsies after flea exposure under the effect of various incubating substances

	Allergic dogs			Non-allergic dogs		
	Compound	Flea antigen	Diluent	Compound	Flea antigen	Diluent
Tryptase	↑ (3.0 vs. 5.6)	n.s. (0.6 vs. 0.4)	n.s. (5.0 vs. 4.0)	n.s. (1.1 vs. 1.5)	n.s. (1.0 vs. 1.5)	↑ (1.0 vs. 5.5)
Chymase	↑ (1.0 vs. 2.7)	n.s. (0.5 vs. 0.4)	n.s. (4.9 vs. 6.0)	n.s. (0.4 vs. 0.9)	n.s. (1.2 vs. 0.9)	↑ (1.0 vs. 2.3)
IL-4	↑ (2.6 vs. 5.8)	↑ (0.7 vs. 2.9)	n.s. (3.6 vs. 13.9)	↑ (2.0 vs. 9.1)	n.s. (7.1 vs. 3.0)	↑ (1.0 vs. 2.3)
IL-5	n.s. (2.0 vs. 3.2)	n.s. (1.6 vs. 1.1)	n.s. (3.2 vs. 12.8)	n.s. (0.4 vs. 0.9)	n.s. (2.8 vs. 1.8)	↑ (1.0 vs. 3.8)
IL-13	n.s. (3.3 vs. 3.4)	↓ (1.2 vs. 0.7)	n.s. (8.6 vs. 13.9)	↑ (0.08 vs. 0.7)	n.s. (1.2 vs. 0.8)	↑ (1.0 vs. 4.2)
TNF-α	↑ (2.4 vs. 4.2)	n.s. (0.4 vs. 0.6)	n.s. (4.8 vs. 8.3)	↑ (0.2 vs. 1.1)	n.s. (1.9 vs. 1.8)	↑ (1.0 vs. 4.9)
IFN-γ	n.s. (1.0 vs. 1.2)	n.s. (0.2 vs. 0.1)	n.s. (4.3 vs. 3.7)	n.s. (0.6 vs. 0.2)	↓ (2.6 vs. 0.3)	n.s. (1.0 vs. 0.6)

↑: significant increase of mRNA production at day 4 compared to day 1; ↓: significant decrease of mRNA production at day 4 compared to day 1; n.s.: no significant change in mRNA production at day 4 compared to day 1; values in parenthesis represent median of relative units (day 1 vs. day 4).

allergic dogs compared to non-allergic dogs (Table 5).

- Comparing the response of the two groups to flea exposure shows that mRNA production of IL-4, IL-5 and IL-13 is significantly increased in allergic dogs, whereas non-allergic dogs do not respond with an increase in cytokine production (Table 4).

4. Discussion

The aim of this study was to investigate the late phase immunological response in allergic and non-allergic dogs that have been exposed to flea antigen in a standardized manner. For this purpose we performed a quantitative analysis of cytokine and MC protease mRNA production in skin biopsies and PBMC and we investigated the inflammatory reaction in the skin. Since in two previous studies the histological reaction

pattern and the expression of MC proteases did not change significantly within the first 4 days of flea exposure, we decided to take the biopsies before and after 4 days of flea exposure (von Ruedorffer et al., 2003; Welle, personal communication).

Our study demonstrates clearly that a significant difference with regard to IDT, inflammation score, MC, IgE+ cells, and eosinophils density as well as cytokine mRNA production in skin biopsies and PBMC exists between allergic and non-allergic dogs. Furthermore, we show that skin biopsies and PBMC respond in a different way before and after in vivo and in vitro stimulation with flea antigen.

The inflammatory response in the skin biopsies to flea exposure was present in non-allergic and allergic dogs. However, in the allergic group the inflammation score was already higher before flea exposure. This finding is in agreement with the two above mentioned previous studies where biopsies from flea allergic, non-allergic and naïve dogs taken before as well as

Table 3

Differences in mRNA levels in skin biopsies between allergic and non-allergic dogs, under the effect of various incubating substances

	Day 1			Day 4		
	Compound	Flea antigen	Diluent	Compound	Flea antigen	Diluent
Tryptase	n.s. (3.0 vs. 1.1)	n.s. (0.6 vs. 1.0)	+ (5.0 vs. 1.0)	+ (5.6 vs. 1.5)	– (0.4 vs. 1.5)	n.s. (4.0 vs. 5.5)
Chymase	n.s. (1.0 vs. 0.4)	– (0.5 vs. 1.2)	+ (4.9 vs. 1.0)	n.s. (2.7 vs. 0.9)	n.s. (0.4 vs. 0.9)	n.s. (6.0 vs. 2.3)
IL-4	n.s. (2.6 vs. 2.0)	– (0.7 vs. 7.1)	n.s. (3.6 vs. 1.0)	n.s. (5.8 vs. 9.1)	n.s. (2.9 vs. 3.0)	n.s. (13.9 vs. 2.3)
IL-5	+ (2.0 vs. 0.4)	– (1.6 vs. 2.8)	+ (3.2 vs. 1.0)	+ (3.2 vs. 0.9)	n.s. (1.1 vs. 1.8)	n.s. (12.8 vs. 3.8)
IL-13	+ (3.3 vs. 0.08)	n.s. (1.2 vs. 1.2)	+ (8.6 vs. 1.0)	+ (3.4 vs. 0.7)	n.s. (0.7 vs. 0.8)	n.s. (13.9 vs. 4.2)
TNF-α	+ (2.4 vs. 0.2)	– (0.4 vs. 1.9)	+ (4.8 vs. 1.0)	+ (4.2 vs. 1.1)	– (0.6 vs. 1.8)	n.s. (8.3 vs. 4.9)
IFN-γ	n.s. (1.0 vs. 0.6)	– (0.2 vs. 2.6)	+ (4.3 vs. 1.0)	n.s. (1.2 vs. 0.2)	n.s. (0.1 vs. 0.3)	n.s. (3.7 vs. 0.6)

+: significantly higher mRNA level in allergic compared to non-allergic dogs; –: significantly lower mRNA level in allergic compared to non-allergic dogs; n.s.: no significant difference in mRNA level in allergic compared to non-allergic dogs; values in parenthesis represent median of relative units (day 1 vs. day 4).

Table 4

Changes in mRNA production in PBMC after flea exposure under the effect of various incubating substances

	Allergic dogs			Non-allergic dogs		
	ConA	Flea antigen	Diluent	ConA	Flea antigen	Diluent
IL-4	↑ (195.1 vs. 352.5)	↑ (30.2 vs. 58.6)	n.s. (5.6 vs. 3.5)	↑ (103.7 vs. 351.7)	n.s. (2.0 vs. 1.5)	↑ (1.0 vs. 1.9)
IL-5	n.s. (20.2 vs. 20.5)	↑ (1.8 vs. 6.7)	n.s. (5.0 vs. 4.1)	↑ (14.6 vs. 51.7)	n.s. (1.9 vs. 1.5)	↑ (1.0 vs. 2.0)
IL-13	↑ (428.3 vs. 672.0)	↑ (6.8 vs. 54.9)	↑ (0.8 vs. 1.1)	↑ (254.3 vs. 1080.4)	n.s. (2.0 vs. 0.8)	n.s. (1.0 vs. 1.2)
TNF-α	n.s. (15.6 vs. 17.1)	n.s. (3.3 vs. 4.6)	n.s. (1.5 vs. 1.2)	↑ (14 vs. 32.6)	n.s. (1.7 vs. 1.6)	↑ (1.0 vs. 2.9)
IFN-γ	↓ (506.5 vs. 293.8)	n.s. (1.3 vs. 1.8)	↓ (1.1 vs. 0.3)	↑ (441.0 vs. 829.0)	n.s. (0.4 vs. 0.4)	n.s. (1.0 vs. 1.0)

↑: significant increase of mRNA production at day 4 compared to day 1; ↓: significant decrease of mRNA production at day 4 compared to day 1; n.s.: no significant change in mRNA production at day 4 compared to day 1; values in parenthesis represent median of relative units (day 1 vs. day 4).

1 and 3 days (1. study) or 4, 21 and 35 days (2. study) after flea exposure were compared (von Ruedorffer et al., 2003; Welle, personal communication).

An important regulator of the allergic response is the cross-talk between eosinophils and MC (Pili-ponsky et al., 2002). During the allergic response, MC are activated by antigen cross-linking of IgE bound to the Fc epsilon RI. In our study, we found a significantly higher number of IgE+ cells in the allergic group before and after flea exposure, which supports the role of IgE in FAD. The cross-linking results in the release of preformed and newly synthesized mediators and the clinical symptoms of the early phase of allergy (Arm and Lee, 1992). A few hours later, a late phase reaction is observed. This reaction and the chronic allergic inflammation are characterized by an infiltration of eosinophils and CD4+ T-cells (Gaga et al., 1991). Eosinophils are crucial for the induction of the inflammatory process and for causing tissue damage (Foster et al., 1996). Therefore, we investigated the number of eosinophils and MC in skin biopsies. Importantly, we found that,

although in both groups the density of eosinophils increased after 4 days of flea exposure, a significant and much stronger increase was found in allergic dogs. This may be of diagnostic value for dermatopathologists, since it suggests that a high number of eosinophils in skin biopsies is compatible with an allergic status in dogs and not only the result of exposure to ectoparasites. A strong eosinophilic infiltration in FAD was also observed by Gross and Halliwell (1985), who found a predominant infiltration with eosinophils for up to 24 h at the site of flea bites or after IDT.

On day 1 before flea exposure the number of MC/mm² was significantly higher in allergic dogs compared to non-allergic dogs. However, after flea exposure, no difference with regard to MC density between the two groups was observed. This is the result of a decrease in MC numbers in the allergic animals whereas MC density stayed about the same in the non-allergic dogs. This finding suggests that in allergic animals MC degranulate more easily after appropriate stimulation and therefore are no longer

Table 5

Differences in mRNA levels in PBMC comparing allergic vs. non-allergic dogs, under the effect of various incubating substances

	Day 1			Day 4		
	ConA	Flea antigen	Diluent	ConA	Flea antigen	Diluent
IL-4	n.s. (195.1 vs. 103.7)	+ (30.2 vs. 2.0)	+ (5.6 vs. 1.0)	n.s. (352.5 vs. 351.7)	+ (58.6 vs. 1.5)	+ (3.5 vs. 1.9)
IL-5	n.s. (20.2 vs. 14.6)	n.s. (1.8 vs. 1.9)	+ (5.0 vs. 1.0)	n.s. (20.5 vs. 51.7)	+ (6.7 vs. 1.5)	+ (4.1 vs. 2.0)
IL-13	n.s. (428.3 vs. 254.3)	n.s. (6.8 vs. 2.0)	n.s. (0.8 vs. 1.0)	n.s. (672.0 vs. 1080.4)	+ (54.9 vs. 0.8)	n.s. (1.1 vs. 1.2)
TNF-α	n.s. (15.6 vs. 14.0)	+ (3.3 vs. 1.7)	n.s. (1.5 vs. 1.0)	n.s. (17.1 vs. 32.6)	+ (4.6 vs. 1.6)	– (1.2 vs. 2.9)
IFN-γ	n.s. (506.5 vs. 441.0)	n.s. (1.3 vs. 0.4)	n.s. (1.1 vs. 1.0)	– (293.8 vs. 829.0)	+ (1.8 vs. 0.4)	– (0.3 vs. 1.0)

+: significantly higher mRNA production in allergic compared to non-allergic dogs; –: significantly lower mRNA production in allergic compared to non-allergic dogs; n.s.: no significant change in mRNA production in allergic compared to non-allergic dogs; values in parenthesis represent median of relative units (day 1 vs. day 4).

detectable. A similar finding is reported in a study where lesional skin of human patients with FAD revealed a low number of MC (Garcia et al., 2004). Furthermore, two studies performed in dogs and guinea pigs show that no increase in MC after flea exposure is found (Johnston and Brown, 1985; von Ruedorffer et al., 2003).

In contrast to the MC density, the number of IgE-bearing cells in allergic dogs did not decrease after flea exposure. This finding may point out either (I) that mast cells are degranulated and thus no longer detectable with toluidine blue staining or (II) that not only MC but also dendritic cells are involved in allergen uptake. It has already been shown by other authors that epidermal (i.e. Langerhans cells) and dermal dendritic cells in the skin of dogs with AD express surface IgE, whereas IgE expression could not be detected in the skin of control dogs (Olivry et al., 1996).

Investigating mRNA production in skin biopsies before flea exposure and without additional in vitro stimulation, we found a higher constitutive mRNA level of all cytokines and proteases tested in allergic than in non-allergic dogs. We chose Ringer's solution as a neutral incubating substance with no effect on the ability of the inflammatory cells to degranulate or to produce and secrete cytokines. However, to rule out the possibility that stimulatory factors, such as lipopolysaccharides were present in our Ringer's solution, we performed a further experiment and investigated the mRNA production in the paraffin-embedded biopsies, which were taken before flea exposure and immediately fixed in formalin. This experiment confirmed the higher constitutive mRNA level in allergic dogs before flea exposure. These results may be explained by the data obtained by the histological examination where a higher inflammation score, higher numbers of MC and IgE+ cells were observed in allergic dogs compared to non-allergic dogs before flea exposure. Exposure to fleas for 4 days resulted in an increase in mRNA production of cytokines and proteases (with the exception of IFN- γ) in skin biopsies of non-allergic dogs. Surprisingly we did not see a similar increase in the allergic group. This resulted in statistically not different mRNA cytokine levels after 4 days of flea exposure in both groups. However, the median values of the data showed markedly higher mRNA levels in allergic dogs. These contradictory results may be explained by

the presence of two non-allergic dogs that had extremely high (extreme outliers) mRNA levels (especially IL-4, IL-5 and IL-13). These findings may suggest that MC are the main source of these cytokines since the MC density is comparable in non-allergic and in allergic dogs at day 4.

In vitro stimulation with whole flea antigen of skin biopsies from allergic dogs before flea exposure resulted in a down regulation of chymase, IL-4, IL-5, TNF- α and IFN- γ mRNA compared to non-allergic dogs. We chose whole flea antigen supplied by Greer since this antigen is well known in the literature and more easy to access. However, we cannot exclude completely that the use of more specific antigens such as flea saliva or Cte f 1 could have had a stimulatory effect. This will have to be tested in a further experiment. After 4 days of flea exposure only an increased production of IL-4 mRNA could be achieved with additional stimulation with flea antigen in skin biopsies of allergic dogs. There are very few data in the literature to explain our results. Koning et al. (1997) demonstrated that IL-5 mRNA levels in unstimulated T-cells in children with non-allergic asthma were negligible, whereas protein levels were significantly increased compared to a healthy control group. Unfortunately, activities in this area are limited in veterinary research because few assays exist to measure canine cytokine proteins.

In contrast to skin biopsies, PBMC from allergic dogs stimulated in vitro with whole flea antigen showed a marked increase of TH2 cytokine mRNA production after 4 days of flea exposure. This contradictory finding might be a result of a different sensitivity of PBMC and inflammatory cells in the skin towards the antigen. PBMC consist of a relatively homogenous cell population, whereas in skin biopsies a heterogeneous cell population and a rather complex microenvironment is present.

In addition to TH2 cytokines, in PBMC IFN- γ was increased in allergic dogs after flea exposure and additional in vitro stimulation with flea antigen. This finding suggests that in allergic dogs cells were stimulated to express a wider spectrum of cytokine mRNA compared to non-allergic dogs. Since naïve T-cells are present in both allergic and non-allergic dogs to a similar amount it is likely that in allergic dogs more specific memory T-cells are present that respond upon stimulation with flea antigen.

After 4 days of flea exposure and additional in vitro stimulation with flea antigen, the mRNA levels of all cytokines tested were significantly higher in allergic dogs compared to non-allergic dogs. In particular, IL-4 and IL-13 were much higher in allergic dogs. The increased expression of IL-13 is in agreement with a study performed in human beings, where IL-13 production by antigen stimulated PBMC of subjects with seasonal allergic rhinitis was markedly stronger than in non-atopic people. However, in the same study a strong correlation with IL-5, but not IL-4 was observed (Li et al., 1998).

To our knowledge, so far no data evaluating quantitative cytokine expression in dogs with allergic dermatitis has been published. Therefore, our results obtained from dogs with FAD are compatible, although more precise, with data from semi-quantitative cytokine mRNA analyses, which have been performed in dogs with AD. Hayashiya et al. (2002) demonstrated that IL-4 and IL-5 mRNA production in freshly isolated PBMC from dogs with AD is higher in allergic compared to non-allergic dogs. In our allergic dogs before flea exposure IL-4 and IL-5 mRNA in PBMC was also higher. Therefore, we conclude that a TH2 polarisation similar to AD occurs in FAD (Nuttall et al., 2002a). Since we also observed an increase of IFN- γ mRNA, which is known to inhibit IgE production (Coffman and Carty, 1986), we suggest that FAD is triggered not by a defective TH1 response but rather by a skewing towards TH2 response.

The human counterpart of canine FAD is a cutaneous hypersensitivity reaction known as papular urticaria, which can be triggered by fleabites. The immunological mechanisms in man underlying the clinical progression of the disease are still mostly unknown and the diagnosis is normally made by clinical assessment. In a recent study, it was shown that the number of MC in patients suffering from papular urticaria was low and that skin lesions were accompanied by a predominance of a T-cell response. The initial clinical lesions (vesicles) seem to be caused by an enrichment of CD8+ T-cells (Garcia et al., 2004). Because of the similar clinical and histopathological findings of FAD in dogs and papular urticaria in human beings, our dogs could represent a useful model for the study of flea-induced papular urticaria. Furthermore, because of the close relationship of the allergic response to the one reported in AD, in our

opinion, this FAD model is also very suitable to further characterize the immune dysregulation in allergic dermatitis in dogs and human beings in general. These dogs could be used as a potential tool to develop therapeutic strategies aimed to control the immune response in allergic dermatitis.

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