

# Monitoring of basophil sensitization to antigens of the cat flea (*Ctenocephalides felis felis*): a new tool for the diagnosis of feline flea bite hypersensitivity?

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**Abstract** Suitability of blood basophils for in vitro diagnosis of flea allergy dermatitis (FAD) or flea bite hypersensitivity was studied in cats. A functional in vitro test (FIT) for sensitized type I allergic effector cells was used to evaluate the degree and kinetics of in vivo basophil sensitization against flea antigens in cats under long-term flea exposure. FIT results were compared with intradermal (IDT) and serological testing. Before, during, and after weekly repeated exposure to *Ctenocephalides felis*; 14 cats

were repetitively FIT-assessed for general and flea-specific sensitization. In three cats, flea-specific sensitization was seen before and throughout flea exposure. Five cats, although generally sensitized, never developed a flea-specific sensitization. Six cats initially FIT-negative became sensitized for flea antigen during flea infestation. Induction, upregulation, and binding of *C. felis*-specific sensitizing antibodies to basophils during flea challenge may explain the developing sensitization in these cats. Strong discrepancies between the levels of flea-specific circulating IgE and basophil sensitization contrasted comparable results for basophil and mast cell sensitization using FIT and IDT, respectively. Hence, the FIT might provide an immunological supplement to the clinical diagnosis of FAD in cats by elucidating the state of basophil-sensitization to flea antigens. And it may be a comfortable alternative to IDT.

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## Introduction

Flea bite hypersensitivity, also known as flea allergy dermatitis (FAD), is one of the most common dermatological disorders in cats in areas where fleas are endemic (Scott et al. 2001). It is a disease in which a hypersensitivity state in a host is produced against antigenic material from fleas mainly in response to the injection of such material from their salivary glands. Yet, in some cases, a hypersensitivity state may perhaps be produced in response to inhalation of cat flea allergens, as cat flea allergens may contribute to the allergenicity of house dust in areas of heavy flea infestation (Trudeau et al. 1993). Type I hypersensitivity reactions are of central importance in the development of clinical signs of flea bite hypersensitivity, and clinical improvement of FAD can only be achieved by preventing the sensitized animal from being bitten by fleas (Carlotti and Jacobs 2000).

Diagnosis of flea bite hypersensitivity is usually based on the patient's history, clinical signs, presence of fleas and/or flea dirt, and response to appropriate anti-flea treatment. Unfortunately, clinical signs of FAD can mimic almost any pruritic dermatosis, and pruritus in the cat is often masked by overzealous grooming instead of scratching (Moriello and McMurdy 1989). Besides, evidence of fleas is not always apparent (Scott et al. 2001). Hence, in order to confirm suspected hypersensitivity reactions against fleas and to distinguish flea bite hypersensitivity from other causes of allergic dermatitis, such as hypersensitivities to various parasites, food, chemicals, or drugs, reliable allergy tests with flea antigens are required.

The available *in vivo* method for determining the presence of sensitizing antibodies is the intradermal skin test (IDT). In several studies, the IDT has been used to evaluate the specificity of other allergy tests for flea bite hypersensitivity in the cat (Bevier et al. 1997; Foster and O'Dair 1993; Slacek and Opdebeeck 1993). Even so, in practice intradermal testing in the cat is often hard to interpret since cats appear to react with poorly defined wheals and little erythema (Kunkle 1984). Furthermore, an *in vivo* test includes allergen boosting and is traumatic for the animal. Moreover, stress-related mediator release might interfere with the development of clear skin reactions (Willemse et al. 1993).

*In vitro* methods are comparatively attractive, as there is no unnecessary allergen boosting; the patient can be tested despite of severe skin disease, and sedation is usually not required. However, studies that compared serological tests with skin tests to document allergic sensitization in dogs, cats, and humans have shown that levels of free, allergen-specific immunoglobulin in serum are not always associated with skin reactions based on allergen-specific antibodies bound to allergic effector cells (Foster and O'Dair 1993; Jackson et al. 1996; Gilbert and Halliwell 1998; Laffort-Dassot et al. 2004; Purohit et al. 2005).

The above-mentioned plus the fact that a proportion of animals (cats and dogs) with clinical signs of FAD fails to show evidence of either flea antigen specific IgE or immediate skin test reactivity has prompted us to search for an alternative and more reliable test methodology. The functional *in vitro* test for sensitized type I allergic effector cells (FIT)—basically developed for horses by Kaul (1998)—provides a new *in vitro* monitoring system for feline allergen-specific antibodies that were bound *in vivo* to sensitize effector cells of type I allergies such as basophilic granulocytes and mast cells (Stuke et al. 2003). To test the existence of allergen-specific, sensitizing antibodies on basophils with the FIT, washed blood cells are incubated *in vitro* with selected antigen. If membrane-bound antibodies on basophils are bridged by the antigen, immediate histamine release will be triggered as a result (specific

sensitization). Histamine is constitutively stored in mast cells and basophils exclusively; consequently, as basophils are the major contributor to blood cellular histamine, the most significant measured histamine in the FIT is of basophil origin. Differential antigen-triggered histamine release permits discrimination between sensitized and non-sensitized animals. In addition, information about total cellular histamine content, spontaneous histamine release and the general ability of basophils to degranulate after cross-linking of membrane-bound antibodies irrespective of their specificity (general sensitization) can be obtained in the FIT.

To investigate if feline basophilic granulocytes provide a suitable source for *in vitro* determination of a functional flea-specific sensitization of cats, we studied (1) the FIT for documentation and evaluation of the kinetics of *in vivo* basophil sensitization against flea antigens in cats under controlled long-term flea exposure and (2) compared the FIT results with those obtained by IDT and by a serological test using the same flea antigen preparation.

## Material and methods

### Cats

This study was performed with 14 laboratory-bred European short-haired cats from the research colony of the Institute of Parasitology, Department of Infectious Diseases, University of Veterinary Medicine Hanover (10 castrated males and four spayed females, aged 4 to 7 years). Each cat was given an identification number (ID) counting 2–13, 15, and 16 (note: cats #1 and #14 had to be excluded from the initially designated study population). Before flea infestations were carried out, all animals were clinically healthy and without known history of skin disease. Dermatophytosis and ectoparasites had been excluded by standard diagnostic procedures (Scott et al. 2001). Prior to this study, the cats had been prevented from flea exposure for at least 1 year. Before controlled flea exposure, all experimental cats were monitored three times (weeks −11, −6, and −3) by means of the FIT and their clinical scoring in order to assess any sign of previous sensitization or disease. During flea infestations the cats were housed indoors in separated, individual cages. Between infestations they were group-housed in groups of three to seven cats. All cats were cared for and housed in facilities according to the principles of the Department of Animal Welfare and Behaviour of the University of Veterinary Medicine Hannover. Since the major interest of this study was to induce and follow the course of sensitization of experimental cats toward fleas, this labor-intensive study design did incorporate as many infested cats as logistically possible, but no uninfested or never infested cats.

## Flea infestations

A laboratory strain of *Ctenocephalides felis felis* (Bouché 1835) routinely maintained on laboratory cats of the Institute of Parasitology, University of Veterinary Medicine Hannover was used. For infestation, unfed fleas that were 3–5 days post-emergence were released over the cats' dorsal midlines and allowed to disperse freely. During infestation cats were allowed to groom themselves normally. Cats were expected to develop sensitization to fleas through repetitive antigen exposure by weekly reapplication of 100 fleas per cat over a period of 28 weeks. After 24 h of flea contact each cat was combed thoroughly over the entire body for at least 20 min with a fine-toothed flea comb to remove fleas. This provided an environment with 6 days of highly reduced flea contact between each re-infestation and prevented accumulation of fleas over time.

## Clinical assessment of cats

Dermatological evaluation of each cat was made once per week throughout the study. For objective assessment of clinical signs, a validated Scoring Index for Clinical Signs of flea allergy dermatitis (FAD) in cats was used (Kunkle et al. 2000). For that, a cat's body was divided into five regions (head/neck, dorsal thorax, ventral thorax/abdomen, lumbar-sacral region/tail, and perineum/hind legs) and dermatological parameters, such as alopecia, crusts, papules or scales, were assessed according to their degree of severity using scores between 0 and 3 (0 meaning no changes and 3 indicating a severely affected skin). To compare different intra- and inter-individual examinations, a total score was calculated per cat after each assessment by summing up the scores of different body regions.

## Blood collection for the FIT

To perform the FIT blood samples were harvested 11, 6, and 3 weeks before flea exposure and biweekly during the study by puncturing the *Vena cephalica antebrachii* of the right or left foreleg without anesthetics. The minimum amount of blood (anticoagulant: K-EDTA) required to carry out the FIT for the studies described was 2.2 ml per cat.

## The Functional in vitro Test for sensitized type I allergic effector cells (FIT)

The FIT was adapted from the procedure developed preferentially for horses by Kaul (1998). As soon as possible after harvest, each blood sample was washed twice with phosphate-buffered saline (PBS) in order to get rid of plasma together with none cell bound antibodies and was finally reconstituted with PBS to its original volume

("washed blood cells"). Subsequently, the FIT was performed without delay by applying each washed blood sample to the following four kinds of treatment: (1) Spontaneous release of mediators by incubating 200 µl of washed blood cells 1:2.5 diluted in releasing buffer (Pipes B) at 37°C for 60 min. (2) Physical release of mediators was obtained by boiling 200 µl washed blood cells 1:5 diluted in Pipes B for 10 min in a water bath. (3) Testing for "general sensitization": To test the occupation of basophils with antibodies—irrespective to their specificity—but able to mediate a degranulation of their cell upon cross-linking by anti-immunoglobulin isotype antibodies, the blood cells were incubated with a suitable rabbit antiserum against heavy and light chains of feline immunoglobulin G (RαC IgG [H+L]) at final concentrations of 100 and 30 µg/ml (anti-immunoglobulin induced mediator release). Either the physical or the anti-immunoglobulin induced treatment served to determine the maximal amount of histamine detectable in the individual sample of washed blood cells. (4) Testing for "flea antigen specific sensitization": Sensitization of basophilic granulocytes with antibodies specific for flea antigens was detected by stimulating blood cells with an aqueous whole flea extract (*Ctenocephalides felis*/ *C. canis*, Greer Laboratories, Lenoir, USA).

In each FIT, seven final concentrations of the flea extract between 1 µg/ml and 1 pg/ml were tested (6 steps of 1:10 serial dilutions). Before application, the aqueous flea extract was purified from preservatives by gel filtration technique using a Sephadex G PD 10 column. Both the anti-immunoglobulin- and the antigen-induced releases were performed by incubating 200 µl of washed blood cells with 300 µl Pipes B containing either antibodies or antigens at 37°C for 60 min. Subsequently, the samples were pelleted and the histamine containing cell and particle-free supernatant was collected and stored at −20°C.

## Quantitative histamine determination

Due to the fact that among all mediators to be released from different blood cells histamine is the only one reliably representing the basophils, the essential type I effector cells in peripheral blood, we quantified histamine in the released supernatants as best read-out parameter for the basophil function. After completing the FIT, a competitive radioimmunoassay (RIA) was used to quantify the amount of released histamine in cell-free supernatant of each sample. The assay included a radioactive histamine tracer to compete with histamine from samples for binding to antihistamine antibodies. First, an acyl group was introduced into sample histamine by means of NHS biotin. Then samples were incubated with an acyl-iodide<sup>125</sup>-histamine tracer and goat anti-N-acyl-histamine antiserum overnight. After 16–20 h, a donkey anti-goat

IgG antiserum was used for precipitation of immune complexed histamine.

Finally, precipitated radioactivity, expressed in counts per minute (cpm), was measured using a gamma counter. Results were corrected for the background of unspecifically precipitated radioactivity. Relative radioactivity values were converted into histamine concentration per milliliter of original blood for each sample using a sigmoid six-point standard curve with a lower detection limit of 0.3 ng histamine per milliliter and an upper limit of 100 ng histamine per milliliter. The proportional histamine content of each test sample was calculated from the maximal histamine content in either the physical or the anti-immunoglobulin release sample of each individual blood sample.

#### Intradermal testing for mast cell reactivity to flea antigens

Intradermal tests were performed on each cat by established methods (Scott et al. 2001) with the same flea antigen preparation used in the FIT (*Ctenocephalides felis/C. canis*, 1:1,000 w/v (= 40 µg/ml), Greer Laboratories, Lenoir, USA) in week 4, 14, and 28 of the study. To perform the IDT, cats were sedated with 6–10 mg/kg of ketamine and 1–2 mg/kg xylazine intramuscularly. After sedation, the lateral thorax was shaved and cleaned. The flea allergen preparation in parallel to the negative (phosphate buffered saline, Greer Laboratories) and positive (histamine phosphate, 27.5 µg/ml, Greer Laboratories) controls were injected intradermally in a volume of 50 µl (=2 µg allergen/injection) using 1-ml tuberculin syringes with 27 gauge needles. Immediate reactions were assessed 15 min after injection. The skin response was recorded as the diameter of the wheal reaction. Positive reactions were characterized by a wheal equal to or larger than the average diameter of the negative and positive control wheals at the time of reading. Observations for a delayed reaction were not a focus of this study. However, it was noted that none of the cats showed delayed skin test reactivity after 24 h.

#### ELISA for determining IgE antibody to flea saliva and flea whole-body extract

To monitor the level of IgE antibody to flea salivary antigens and flea whole-body extract, respectively, approximately 0.5 ml of serum was collected from each cat at weeks -3, 14, and 28 and frozen at -80°C before being submitted for measurement using the Allerecept® test. This test is a non-competitive ELISA kindly performed by Heska, Fribourg, Switzerland. The assay system used 1:6 diluted cat serum and utilized a biotinylated recombinant extracellular domain of the human Fcεpsilon receptor I alpha-chain (FcεRIα) as the primary tracer for allergen-specific IgE molecules. Streptavidin alkaline phosphatase

was used as the enzyme containing secondary tracer and *p*-nitrophenyl phosphate as substrate. Plates were coated with either a preparation of Greer flea whole-body extract (4 µg/ml) or a mixture of native flea saliva (1.25 µg/ml) and a recombinant protein of the flea salivary antigen Cte f 1 (0.625 µg/ml) (McCall et al. 1997; Stedman et al. 2001). The test was considered positive when the titres were ≥150 EA units (cutoff determined by Heska Corp.).

#### Data treatment

The relative amount of histamine released with different treatments (spontaneous, anti-immunoglobulin-induced, antigen-induced) of each blood sample was calculated by comparison to the maximal amount of histamine (nanogram histamine per milliliter blood) available in the blood at each sampling. Relative antigen-induced histamine values >100% can be explained by a loss of histamine during physical treatment of cells. Absolute (nanogram histamine per milliliter blood) and relative (histamine as percentage of the corresponding maximal histamine release) values of histamine releases were calculated using Microsoft Excel 10.0.

Using relative spontaneous histamine releases from 226 samples of the experimental cats, an exclusion criterion for samples was determined as three standard deviations (SD) above the mean of the spontaneous releases. This criterion was used to prevent evaluation of samples with pre-damaged or pre-stimulated basophils showing un-physiologically high spontaneous releases.

After exclusion of technically questionable samples, the mean of all spontaneous histamine releases ( $n=195$ ) was determined. A reliable evaluation of antigen-induced histamine releases without interference of high spontaneous releases is given by setting the cutoff at the mean plus 6 SD of the spontaneous releases:  $[4.2\% (\text{Mean}) + 6 \times 2.65\% (\text{S.D.}) = 20.1\%]$ . Subsequently, antigen-induced releases of ≥20% of the corresponding maximal histamine release were defined as positive reaction and those cats were classified as carrying sensitized basophils.

The degree of sensitization of a cat was defined as the lowest allergen concentration necessary to induce a positive histamine release. As seven concentrations of the flea antigen preparation were tested, the degree of sensitization could range between 0 (no positive reaction induced by the flea extract), 1 (positive histamine release induced by an allergen concentration of 1 µg/ml), and 7 (positive histamine release induced by an allergen concentration of 1 pg/ml).

Linear regression analysis was used to evaluate correlations between the time of flea exposure and relative flea antigen-induced histamine releases through a concentration of 1 µg/ml flea antigen preparation.

Wilcoxon signed rank test was used to evaluate differences between the amounts of histamine in blood (nanogram per milliliter) pre and post flea exposure.

McNemar statistics was used to test the difference between results of FIT and IDT or FIT and Allercept® IgE test. To determine the amount of agreement between test results, the simple kappa correlation coefficient ( $\kappa$ ) were calculated. All statistics were performed using SPSS for Windows, version 10.0. Results were considered significant when  $p < 0.05$ .

## Results

In vitro test of feline basophils for their functional sensitization with specific antibodies against cat flea extract harvested from cats before, during, and after controlled long-term flea exposure

Figure 1 shows that three (cats #5, #7, #9) out of the 14 cats reacted positively (histamine release  $\geq 20\%$  of the relevant maximal release) against flea antigens prior to the envisaged experimental flea exposure. The lowest concentration of flea extract able to induce a positive reaction by basophils of these cats was  $0.1 \mu\text{g/ml}$  (graded as 2+). Basophils from 11 cats showed no release upon stimulation with flea antigen used at the same concentrations under identical conditions.

After 28 weeks of repeated weekly exposure with 100 *C. felis* per cat, the status of functional sensitization of some of these 14 cats had changed considerably (Fig. 2): The three presensitized cats (#5, #7, #9) showed either the same (#5=2+), a modestly (#9=3+), or a strongly enhanced (#7=5+) degree of sensitization of their basophils. Six (#2, #8, #10, #11, #12, and #15) out of the 11 cats that were initially negative had developed a positive functional sensitization (1+ to 2+), while five cats (#3, #4, #6, #13, and #16) remained without a functional sensitization of their basophils.

More detailed FIT results listed in Table 1 provide additional information about the basophil reaction pattern of these 14 cats before (week -3) and after 28 weeks of weekly flea exposure: Based on their specific sensitization against flea antigen cluster analysis revealed clearly three groups of cats, group I being constantly sensitized (#5, #7, #9), group II being constantly negative (#3, #4, #6, #13, #16), and group III (#2, #8, #10, #11, #12, #15) being initially negative and becoming positive during flea exposure (see also Figs. 1 and 2). Of interest are two additional parameters: While the maximal detectable histamine content in basophils of group III was comparable before ( $23.4 \text{ ng/ml}$  of blood) and after repeated flea exposure ( $29.8 \text{ ng/ml}$ ), it increased considerably in group I (from  $31.9$  to  $75.8 \text{ ng/ml}$ ) and group II ( $17.4$  to  $45.2 \text{ ng/ml}$ ), indicating that the number of basophils and/or their content of histamine has increased to a similar extent, although no

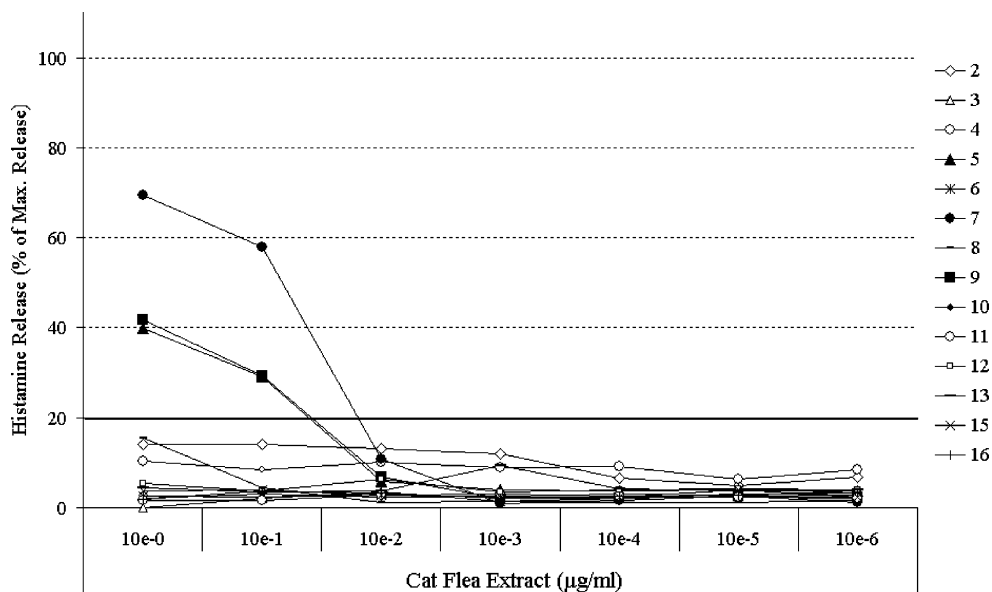
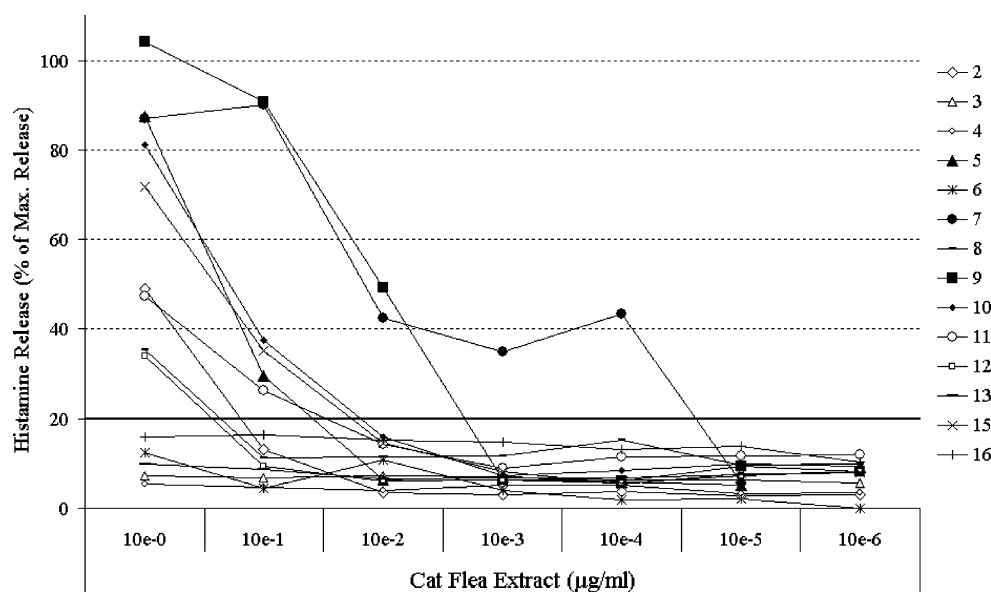


**Fig. 1** Functional sensitization of basophils with specific antibodies against cat flea extract harvested from 14 cats before controlled flea exposure. Individual values for all cats are expressed as dose-dependent percentage of released histamine as compared to the relevant maximal release. Histamine release of  $\geq 20\%$  indicates a positive reaction (**bold horizontal line**). The degree of sensitization of a cat was defined as the lowest allergen concentration necessary to

induce a positive histamine release. Using seven tenfold dilutions of flea extract, the degree of sensitization could range from 0 (no sensitization) and 1 (positive reaction achieved with  $1 \mu\text{g/ml}$ ) up to 7 (positive reaction induced by  $1 \text{ pg/ml}$ ). Notice positive reactions (2+) were seen in cats no. 5, 7, and 9 for the two highest concentration of flea extract

**Fig. 2** Functional sensitization of basophils with specific antibodies against cat flea extract harvested from 14 cats after 28 weeks of controlled weekly flea exposure. Individual values for all cats are expressed as dose-dependent percentage of released histamine as compared to the relevant maximal release. Histamine release of  $\geq 20\%$  indicates a positive reaction (**bold horizontal line**). Now nine (#2, #5, #7, #8, #9, #10 #11, #12, and #15) out of 14 cats were functionally sensitized against flea extract



specific sensitization was detectable in group II in contrast to group I. However, some change in general sensitization (detectable in the FIT by triggering with anti-isotype antibodies, here anti-feline IgG) has occurred in group II: The rather low level of general sensitization before exposure (50.2%) elevated to 98.3% after 28 weeks of flea exposure. This may be of interest when discussing reasons for the lack of flea-specific functional sensitization of that group.

Kinetics of flea antigen-specific sensitization of basophils in vivo under the influence of weekly flea exposure of cats

The FIT allowed a subtle follow up monitoring of basophil reactivity to flea antigen under the influence of weekly flea exposure by means of the amount of released histamine. Applying linear regression analysis to the amount of histamine released from basophils upon triggering with 1 µg of flea extract/ml, the three groups of cats revealed informative results: There was a positive linear relationship between weeks of flea infestation ( $x$ ) and antigen-induced histamine release ( $y$ ) for all three groups. Constantly sensitized animals of group I showed a highly significant increase in antigen-induced histamine release in the order of 1% histamine per week ( $y=1.08x + 59.55$ ;  $r=0.51$ ;  $p<0.001$ ). Likewise, cats of group III that became sensitized only during weekly repeated flea infestation developed a highly significant increase in histamine release ( $y=1.03x + 7.79$ ;  $r=0.64$ ;  $p<0.001$ ) in the same order of magnitude (1% histamine per week). Even cats of group II that reacted constantly negative (below 20% of the individually maximal cellular histamine) to the flea antigen displayed a significant relationship ( $y=0.092x + 4.96$ ;  $r=0.27$ ;  $p<0.05$ ); however, the increase of flea antigen-induced histamine

release in these negative cats was merely 0.1% per week and far below the level of being positive.

Following up the influence of weekly flea exposure on flea-specific sensitization of basophils in vivo peripheral blood of all 14 cats were subjected biweekly to functional in vitro testing (FIT) including seven tenfold dilutions of flea extract in final concentrations of 1 µg/ml down to 1 pg/ml. The degree of flea specific sensitization was determined by the lowest concentration of flea antigen still capable of triggering a histamine release  $\geq 20\%$  of the relevant maximal histamine of each blood sample at each time of harvest.

Figure 3 shows the kinetics and degree of flea antigen-specific basophil sensitization during weekly flea exposure for 28 weeks in 14 cats summarized according to their pattern of sensitization (groups I to III plus cat #8). Except cat #8, which showed flea-specific sensitization already 2 weeks post starting controlled flea exposure and maintained it throughout the experiment, most of the cats did not alter their pre-infestation state of sensitization during the first 14 weeks of controlled flea exposure. Three cats (group I) retained their degree of sensitization between 2 and 3; while 10 cats (group II and III) did not show basophil sensitization for flea antigen. Interestingly, after 16 to 24 weeks of repeated weekly flea exposure flea-specific basophil sensitization became detectable in five out of ten previously negative cats (group III). Their mean degree of flea-specific sensitization was repeatedly between 1 and 2 until the end of the study. Even in cats of group I that reacted constantly positive against flea antigen, an increase in specific sensitization was found after 24 weeks of flea exposure, with degrees of sensitization ranging mostly between 3 and 4 (maximum 6). Five cats (group II), however, never showed a functional sensitization for flea antigen.

**Table 1** FIT results of cats grouped according to the reaction pattern of their basophils to flea antigen before (−3) and after 28 weeks of controlled weekly flea exposure

| Histamine Release: | Cat ID | Week | Maximal (ng/ml)  | Spontaneous (%) | Anti-IgG induced (%) | Flea antigen-induced (%) <sup>a</sup> |                   |                  |                  |                  |                  |                  |                  |
|--------------------|--------|------|------------------|-----------------|----------------------|---------------------------------------|-------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|                    |        |      |                  |                 |                      | Concentration (μg/ml)                 |                   |                  |                  |                  |                  |                  |                  |
|                    |        |      |                  |                 |                      | 100                                   | 10 <sup>-0</sup>  | 10 <sup>-1</sup> | 10 <sup>-2</sup> | 10 <sup>-3</sup> | 10 <sup>-4</sup> | 10 <sup>-5</sup> | 10 <sup>-6</sup> |
| Group I            |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |
| 5                  | −3     |      | 31.9 (26.9;37.9) | 2.8 (0.5;4.6)   | 95.2 (74.6;100)      | 58.0 (35.4;79.8)                      | 47.4 (25.6;69.2)  | 6.7 (3.3;15.1)   | 2.1 (0.5;5.3)    | 2.0 (0.3;3.9)    | 2.6 (0.5;3.7)    | 1.7 (0.4;3.1)    |                  |
| 7                  |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |
| 9                  | 28     |      | 75.8 (37.1;96.8) | 2.2 (1.1;5.8)   | 87.4 (76.7;94.1)     | 91.1 (87.3;100.4)                     | 95.1 (83.3;101.8) | 49.3 (31.8;81.9) | 6.3 (5.9;44.8)   | 9.1 (15.2;7.5)   | 9.2 (6.2;16.4)   | 6.4 (5.4;10.9)   |                  |
| (N=3)              |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |
| Group II           |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |
| 3                  | −3     |      | 17.4 (8.8;29.9)  | 2.9 (1.3;6.1)   | 50.2 (24.7;100)      | 3.4 (1.4;7.4)                         | 3.8 (1.9;8.3)     | 3.6 (2.7;5.9)    | 3.8 (1.1;8.9)    | 3.3 (2.2;9.2)    | 4.5 (3.1;10.4)   | 4.1 (3.3;9.2)    |                  |
| 4                  |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |
| 6                  | 28     |      | 45.2 (29.4;55.7) | 3.8 (2.4;4.9)   | 98.3 (84.1;100)      | 7.4 (5.9;9.3)                         | 7.7 (5.3;9.4)     | 6.9 (4.7;8.1)    | 6.9 (5.6;7.7)    | 5.7 (4.9;6.7)    | 6.4 (3.9;7.2)    | 7.8 (5.1;8.8)    |                  |
| 13                 |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |
| 16                 |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |
| (N=5)              |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |
| Group III          |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |
| 2                  | −3     |      | 23.4 (11.5;38.8) | 2.2 (0.0;2.9)   | 88.4 (78.6;100)      | 2.7 (1.6;5.4)                         | 3.0 (0.3;4.1)     | 2.6 (0.0;4.8)    | 2.4 (0.4;3.0)    | 2.5 (2.1;3.1)    | 2.4 (0.0;3.7)    | 2.3 (0.0;3.9)    |                  |
| 8                  |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |
| 10                 | 28     |      | 29.8 (21.5;57.2) | 3.4 (1.9;5.6)   | 86.7 (75.7;97.5)     | 36.4 (33.8;50.6)                      | 15.9 (9.3;26.2)   | 6.0 (4.2;12.8)   | 7.5 (3.9;8.7)    | 5.4 (4.0;7.8)    | 7.8 (3.4;10.2)   | 5.0 (4.7;8.19)   |                  |
| 11                 |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |
| 12                 |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |
| 15                 |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |
| (N=6)              |        |      |                  |                 |                      |                                       |                   |                  |                  |                  |                  |                  |                  |

Values are expressed as median; numbers in brackets are 25% and 75% percentiles, respectively.

<sup>a</sup> Flea antigen concentrations that induced a histamine release of or above 20% of their relevant maximal release were defined as positive (numbers in bold).

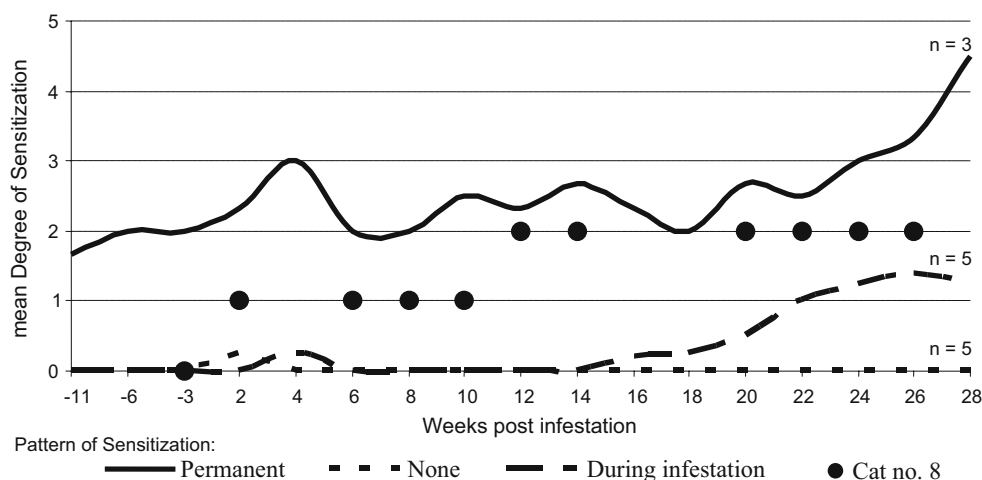


**Fig. 3** Kinetics and degree of flea specific basophil sensitization of 14 cats during 28 weeks of weekly controlled flea exposure. Time dependent degrees of individual sensitization are summarized as mean degree of sensitization for the members of each of the groups I (permanently sensitized cats,  $n=3$ , continuous line), II (non-sensitized cats,  $n=5$ , dotted line), and III (cats developing sensitization during flea infestation,  $n=5$ , broken line). Although associated to reaction

group III, cat #8 was the only one to show flea antigen-specific functional sensitization already 2 weeks after controlled flea exposure. Thus, it reacted much faster than the other five cats of that group where flea-specific sensitization was not seen before 16 weeks of flea exposure. Therefore, it is not included in the mean of group III and is documented separately in this figure (filled dots)

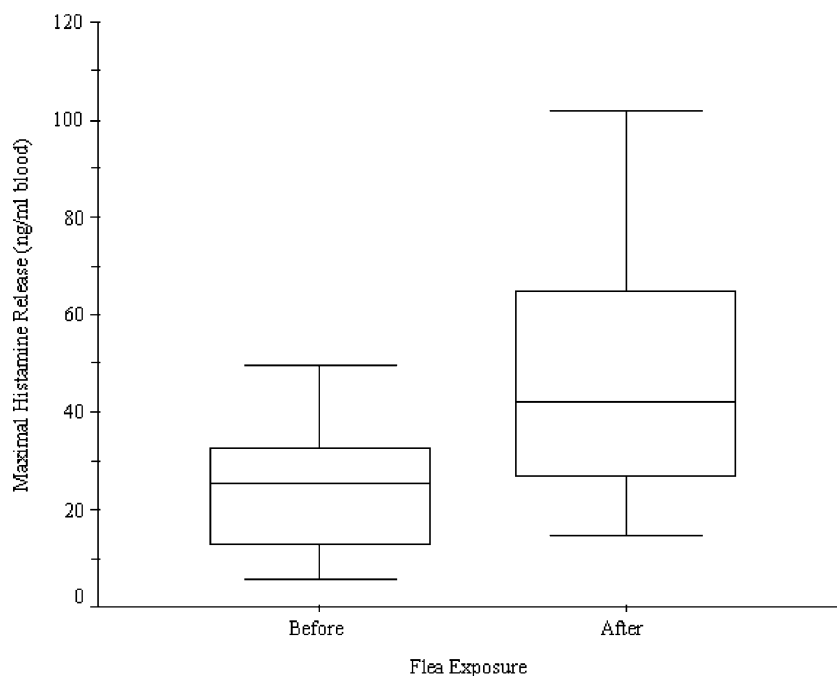
Influence of repeated weekly flea exposure of cats on their histamine content in peripheral blood basophils

In peripheral blood, basophils are the only cells harboring preformed histamine in their granules, which can rapidly be released by either suitable triggering or damaging of these cells. By careful washing of blood just before investigating

the cells, any free histamine in the plasma is eliminated. Thus, any histamine detected upon short-term (1hour) treatment of washed blood cells must have been derived from basophils exclusively.

By comparing the maximal amount of histamine from washed peripheral blood of 14 cats (Fig. 4) before flea exposure (median 25.5 ng/ml of blood, range 5.5–49.0 ng/ml),

**Fig. 4** Box plots summarizing values (ng/ml blood) of total histamine content in basophils of washed peripheral blood harvested from 14 cats 3 weeks before and 28 weeks after weekly controlled flea exposure. Fifty percent of the data are within the box. The horizontal bar within the box represents the median. The upper and lower horizontal lines outside the box indicate the extreme values. Note the significant increase of histamine content per ml of blood ( $p<0.05$ ) after flea exposure as compared to the values before flea exposure



there was a significant ( $p < 0.05$ ) increase in the same cats after 28 weeks of weekly exposure to flea (median 42.1 ng/ml of blood, range 14.7–102.0 ng/ml). The stability of the basophils, however, was not changed as indicated by a constant level of usually less than 6.1% of spontaneous histamine release in the FIT before and after long-term flea exposure (Table 1).

#### Clinical reactions

At the beginning of the study (week 0), all cats were clinically healthy with FAD index scores of 0. During the course of the study only one cat (# 2) developed clear signs of FAD with moderate, fairly generalized miliary dermatitis as of week 26. This cat had the highest clinical score of 15 and was positive both for FIT and IDT from week 14 onward. Scores of the other cats did not exceed 3 during the entire experiment. Symptoms that could temporarily be observed in nine other cats (# 4, # 8, # 9, # 10, # 11, # 12, # 13, # 15, # 16) were confined to broken hairs and a few, isolated crusts. In cat # 7, mild, spot-like alopecia in the shoulder/neck region was seen between weeks 4 and 14.

#### Poor agreement between flea-specific serum IgE levels and flea-specific functional sensitization of basophils

At three time points, 3 weeks before flea exposure, after 14, and after 28 weeks of weekly flea infestation, serum was harvested from all 14 cats for monitoring of flea-specific IgE-antibodies in Allercept®-ELISAs, EDTA-blood was taken for monitoring flea-specific sensitization of basophils by means of the FIT, and an IDT was performed for monitoring flea-specific sensitization of dermal mast cells. Data comparing FcεRIα-based ELISA (Allercept®) results using both the Heska native plus recombinant flea salivary antigens (FSA + rFSA) and the Greer flea whole-body extract (WBE) with FIT results applying Greer whole flea extract as antigen source are given in Table 2.

Comparing the reaction pattern of serum IgE with two different preparations of flea antigen, a mixture of FSA + rFSA (the usual flea antigen source of Heska in their Allercept®-ELISA) and WBE (commercially available from Greer), we found substantial discordances with sera from 13 informative cats. There was very little correlation before flea exposure ( $r^2 = 0.034$ ). After 14 and 28 of weekly flea exposure it had improved, however, only to  $r^2 = 0.321$ . Apparently, the source of flea antigen preparation was very influential on the outcome of the results, even for otherwise identical assays. That was confirmed by comparing the reaction pattern of serum IgE antibodies with sensitizing antibodies on basophils: At no time point was there any significant correlation between FIT results (obtained with flea antigen from Greer: WBE) and ELISA results using

FSA + rFSA from Heska. Therefore, it was of interest to compare the reaction pattern of free serum IgE and cell-bound sensitizing antibodies on basophils using the identical flea antigen preparation from Greer (WBE). However, the results provided no significant correlation between serum IgE reaction and functional properties of sensitizing antibodies on basophils before and after 14 weeks of flea exposure (lower part of Table 2). Interestingly, after 28 weeks of weekly flea infestation, there was a significant correlation between FIT and ELISA results, providing a kappa index value of 0.65 or a correlation coefficient of  $r^2 = 0.480$ . Thus, even with the same antigen preparation free serum IgE antibodies provided very different results than the cell-bound sensitizing antibodies on the basophils.

#### Good agreement between basophil and mast cell sensitization using identical flea antigen preparation

Table 2 summarizes also the results of FIT and IDT obtained with an identical flea antigen preparation (WBE: whole-body extract from Greer) at the same time points: before, after 14 and 28 weeks of weekly flea infestation. Interpretation of the IDT was possible in all cases. No adverse reactions associated with the IDT were seen. Cats were not skin-tested before the beginning of the study because of the concern that it could sensitize their basophils independently from natural sensitization via flea bites. Instead, the first skin tests were done at week 4. In all constantly FIT-positive cats (group I), the results of the IDT were positive at the times of testing as well. At all times of testing, four out of five FIT-negative cats (group II) had also negative IDT results. In five of the six cats that developed basophil sensitization during flea exposure (group III), the FIT and IDT results were negative at weeks 4 and 14. However, after 28 weeks of flea exposure, the FIT results were positive in all six cats, whereas the IDT results were positive only in three of these cats. At weeks 4 and 14 the agreement between FIT and skin test reactions was almost complete or 84% greater than that expected by chance alone (simple  $\kappa = 0.84$  and  $0.84$ , respectively;  $p = 0.001$ ). However, at week 28 the agreement between FIT and IDT results was no more significant.

## Discussion

This study was carried out to investigate if feline basophilic granulocytes provide a suitable tool for the in vitro diagnosis of flea bite hypersensitivity in cats. For that reason, two aims were pursued: (1) the FIT was used for documentation of the degree and kinetics of in vivo basophil sensitization against flea antigens in cats under

**Table 2** Results of four different tests for detection of either specific sensitization of basophils (FIT) or mast cells (IDT) or free serum IgE-antibodies (ELISA) against *C. felis* before<sup>b</sup>, after 14 and after 28 weeks of weekly flea exposure in 14 cats

| Cat ID                            | Before (Week -3)  |                            |                                       | Week 14                    |                            |                  | After (Week 28)                       |                            |                     |                  |                                       |                                |
|-----------------------------------|-------------------|----------------------------|---------------------------------------|----------------------------|----------------------------|------------------|---------------------------------------|----------------------------|---------------------|------------------|---------------------------------------|--------------------------------|
|                                   | FIT <sup>a</sup>  | IDT <sup>a,b</sup>         | FSA + rFSA1<br>ELISA(OD) <sup>c</sup> | WBE-ELISA(OD) <sup>c</sup> | FIT <sup>a</sup>           | IDT <sup>a</sup> | FSA + rFSA1<br>ELISA(OD) <sup>c</sup> | WBE-ELISA(OD) <sup>c</sup> | FIT <sup>a</sup>    | IDT <sup>a</sup> | FSA + rFSA1<br>ELISA(OD) <sup>c</sup> | WBE-<br>ELISA(OD) <sup>c</sup> |
| 2                                 | —                 | —                          | 383                                   | 339                        | —                          | —                | 2,949                                 | 1,134                      | +                   | +                | 2,869                                 | 1,262                          |
| 3                                 | —                 | —                          | 2,256                                 | 26                         | —                          | —                | 2,953                                 | 74                         | —                   | —                | 3,069                                 | 105                            |
| 4                                 | —                 | —                          | 2,916                                 | 306                        | —                          | —                | 3,035                                 | 936                        | —                   | —                | 2,839                                 | 895                            |
| 5                                 | +                 | +                          | 2,907                                 | 384                        | +                          | +                | 2,962                                 | 1,489                      | +                   | +                | 2,960                                 | 771                            |
| 6                                 | —                 | +                          | 54                                    | 2,480                      | —                          | +                | 325                                   | 2,007                      | —                   | +                | 269                                   | 2,385                          |
| 7                                 | +                 | +                          | 134                                   | 2,785                      | +                          | +                | 2,828                                 | 1,901                      | +                   | +                | 2,902                                 | 2,898                          |
| 8                                 | — <sup>(+B)</sup> | +                          | 16                                    | 1,478                      | +                          | +                | 65                                    | 2,037                      | +                   | +                | 3                                     | 1,628                          |
| 9                                 | +                 | +                          | 261                                   | 527                        | +                          | +                | 831                                   | 919                        | +                   | +                | 518                                   | 1,324                          |
| 10                                | —                 | —                          | 70                                    | 1,286                      | —                          | —                | 2,869                                 | 2,308                      | +                   | —                | 2,953                                 | 1,183                          |
| 11                                | —                 | —                          | n. d.                                 |                            | —                          | —                | n. d.                                 |                            | +                   | —                | n. d.                                 |                                |
| 12                                | —                 | —                          | −11                                   | 8                          | —                          | —                | 2,857                                 | 871                        | +                   | +                | 2,905                                 | 1,061                          |
| 13                                | —                 | —                          | 64                                    | 119                        | —                          | —                | −11                                   | −3                         | —                   | —                | −16                                   | 5                              |
| 15                                | —                 | —                          | −9                                    | 1,678                      | —                          | —                | 2,976                                 | 610                        | +                   | —                | 2,562                                 | 909                            |
| 16                                | —                 | —                          | −10                                   | 14                         | —                          | —                | −5                                    | 87                         | —                   | —                | −6                                    | 28                             |
| <i>P</i> value (simple $\kappa$ ) |                   |                            |                                       |                            |                            |                  |                                       |                            |                     |                  |                                       |                                |
| <b>FIT by IDT:</b>                |                   | 0.001 (0.84 <sup>d</sup> ) |                                       |                            | 0.001 (0.84 <sup>d</sup> ) |                  |                                       |                            | n.s. <sup>(e)</sup> |                  | n.s.                                  | 0.012(0.65 <sup>f</sup> )      |
| <b>FIT by ELISA:</b>              |                   | n.s.                       | n.s.                                  | n.s.                       |                            |                  | n.s.                                  | n.s.                       |                     |                  | n.s.                                  |                                |

FIT functional in vitro test for sensitized type I allergic effector cells; FSA + rFSA1 Heska native flea saliva antigens and recombinant flea saliva antigen, IDT Intradermal Test,  $\kappa$  Kappa, n.d., not done, n.s. not significant, OD optical density, WBE Greer flea whole-body extract

<sup>a</sup> With Greer flea whole-body extract

<sup>b</sup> IDT was first performed after 4 weeks of weekly flea infestation

<sup>c</sup> Values reported in bold were considered positive ( $>150$  OD)

<sup>d</sup> Agreement almost complete

<sup>e</sup> Simple  $\kappa$  values are given only if  $p$ -value $<0.05$

<sup>f</sup> Agreement strong

<sup>g</sup> FIT result after 4 weeks of flea exposure, at the time when the IDT was performed

experimentally controlled long-term flea exposure and (2) the results of the FIT were compared with those of IDT and a serological test determining the titre of flea-specific free IgE antibodies in the serum.

Before and during weekly repeated antigen exposure (approximately 100 *C. felis* per cat), 14 cats were repetitively assessed in the FIT for flea antigen-specific sensitization (antigen-induced histamine release), general sensitization (antiimmunoglobulin-induced release), total histamine content, and spontaneous histamine release of their peripheral blood basophils.

General sensitization was seen in all cats when incubating washed blood cells with a predetermined suitable quantity of anti-cat-IgG. Thus, basophils of all cats were sensitized with functional antibodies able to mediate a triggering of histamine release upon bridging. However, a precondition for histamine release from basophils challenged with antigen is the cross-linking of antigen-specific sensitizing antibodies bound in a sufficient density to Fc receptors on the surface of basophils (Ishizaka and Ishizaka 1977). Therefore, antigen-induced histamine releases of basophils in vitro detected by the FIT mirror the presence of an adequate density of antibodies against flea antigens that were bound on the basophil surface in vivo. Flea antigen-induced positive histamine release occurred in three cats before and constantly during experimental flea exposure. Furthermore, at the end of 28 weeks of infestation there was a trend toward a greater percentage of histamine released in response to lower concentrations of the allergen preparation in these cats. This increase in the degree of sensitization suggests that an enhanced number of cross-links were formed on the basophil surface with lower concentrations of antigen. This may be a result of an increased density of *C. felis*-specific cell surface antibodies bound to activating Fc receptors and/or an increased number of basophils having gained sufficient flea-specific sensitization during flea exposure. In addition, increased sensitization to antigen could also reflect qualitative changes in plasma antibodies, e.g., a correlation between the affinity of cell surface antibodies for the antigen and basophil sensitization was shown for human basophils (Mita et al. 2000). Yet, of particular interest are six cats that were FIT-negative in the beginning but became sensitized during the course of flea exposure. In five of these six cats, sensitization could not be detected earlier than 16–24 weeks after initial flea exposure. Only one cat reacted positive as early as of week 2. Infestation-related induction, upregulation, and binding of *C. felis* specific antibodies to Fc receptors on basophils during flea challenge in vivo may explain the development of sensitization in these cats. On the other hand, in five cats that never developed measurable histamine release in the FIT cross-linking may not have occurred to a sufficient

extent. This might be interpreted as either a lack of sensitizing antibodies on the basophil surface or a dominating expression of inhibitory Fc receptors (equivalent to Fc $\gamma$ RIIb in humans; (Kepley et al. 2004)) plus their binding of *C. felis*-specific antibodies.

Interestingly, cats that were constantly FIT-negative (group II cats) had a lower median maximal and median anti-IgG induced histamine release before flea exposure (week -3) than cats that reacted constantly positively (group I cats) or that became sensitized during the course of flea exposure (group III cats) (Table 1). However, this difference cannot sufficiently explain the fact that these cats have never developed a positive histamine release, as their maximal histamine content has more than doubled (from 17.4 to 45.2 ng/ml) and even exceeded that of the FIT positive group III (29.8 ng/ml) at the end of 28 weekly flea exposure. Moreover, the initially comparably low degree of general sensitization (50.2%) went fully up (with 98.3%) to the levels of the FIT-positive groups I and III. Thus, there were sufficient antibodies bound to activating Fc receptors on the basophils of all five cats of the constantly FIT-negative group II (range of general sensitization 84.1 to 100%, Table 1). The finding that at least three (#3, #4, #6) of these five cats had positive titres of IgE antibodies with specificity for flea antigens in their serum (Table 2) leaves at least two possibilities: Either these antibodies did not bind in functionally sufficient amounts to the basophils (even within 28 weeks) that no effective bridging could occur or they had bound to basophils, however, a majority of them to inhibitory Fc receptors. Thus, flea antigen submitted in FIT assays did bind these basophil-bound antibodies but bridged dominantly inhibitory Fc receptors with activating ones prohibiting an activation of basophils and, thus, no histamine release occurred.

Whereas the degree of sensitization reflects the antigen concentration required to induce degranulation, the potential strength of antigen-induced histamine release was reflected by the percentage of histamine released in response to one concentration of flea antigen (1  $\mu$ g/ml). In all donor cohorts (groups I to III), summarized according to their pattern of sensitization, there was a significant relationship between the number of weeks of flea exposure and the percentage of histamine released in response to flea antigen. However, in sharp contrast to the combined data of FIT-negative cats (group II), augmentation of antigen-induced histamine release was ten times stronger in constantly sensitized cats (group I) and those that developed basophil sensitization (group III). Given that flea infestation leads to upregulation of antigen-specific sensitizing antibodies, it is likely that increased binding of sensitizing antibodies to basophils contributed to enhanced strength of histamine release during flea exposure as well. For example, in experimental mouse models, it could be

shown that IgE binding to FcεRI on mast cells and basophils results in increased FcεRI expression, which in turn enhances IgE-dependent mediator release (Kubo et al. 2001; Lantz et al. 1997).

A significant increase in the total blood cellular histamine content was seen in experimental cats with flea exposure. The observed augmentation of total histamine may represent increased basophil counts and/or could be attributable to increased histamine content per cell. However, increased total cellular histamine content of blood did not contribute to the development of clinical allergic conditions of cats in this study, since most animals remained clinically healthy. Thus, a sufficient sensitization of type I effector cells, such as basophils and mast cells is essential but not sufficient for developing clinical symptoms of a type I allergy such as FAD. The development of clinical signs can result from antigen exposure, immediate hypersensitivity, and possibly cutaneous basophil hypersensitivity. Since all are independent variables in the generation of clinical disease, this could explain the lack of considerable clinical symptoms in cats with positive FIT and skin test results.

A further aim of this study was to compare the results of the FIT with those of IDT and a serological test. It was not possible to evaluate the sensitivity and specificity of the FIT in respect to clinical signs because the majority of cats used in this study did not show signs associated with FAD. Likewise, Moriello and McMurdy (1989) found that >30% of cats in a flea-infested environment are sensitized against flea antigens, while clinically normal without signs of flea-derived dermatitis. The facts that the cats used in this study were not selected for a predisposition to flea bite hypersensitivity and were experimentally, rather than naturally, exposed to fleas in order to achieve sensitization, might have had an influence on the development of clinical signs. The time of exposure was based on a previous report that revealed 150 days to be adequate for the development of FAD in experimentally infested, susceptible cats (Colombini et al. 2001). Yet, cats that are without clinical signs despite functional sensitization are of interest, as it may suggest that immunoregulatory mechanisms exist that prevent clinical signs of allergy in spite of a detectable sensitization of the relevant effector cells. There is clearly a need for further investigations of the nature of factors that result in the development of allergy vs clinical non-reactivity. After all, assuming that specifically sensitized cats are basically able to develop flea bite dermatitis after appropriate allergen exposure, the detection of cats with sub-clinical hypersensitivity might be useful with regard to effective flea control strategies.

Potential gender differences could not be detected in this study. Likewise in literature no age or sex predilections have been reported of flea bite hypersensitivity in the cat (Scott et al. 2001).

These cats were not skin-tested before the beginning of the study to avoid sensitization of their basophils or mast cells prior to sensitization via flea bites. For this reason, IDT was performed first at week 4. At weeks 4 and 14 of flea-exposure, an almost complete agreement was found between in vitro basophil degranulation (FIT) and in vivo skin sensitivity (IDT) using identical whole flea extract. However, after 28 weeks of flea-exposure three cats developed basophil sensitization (FIT) but no mast cell sensitization (IDT). Hence, basophil sensitization appears to emerge earlier than mast cell sensitization and might therefore be a more sensitive indicator for a functional type I sensitization. Different responses of sensitized basophils and mast cells to antigen can depend on a great variety of factors. One possibility for the discrepancy between mast cell and basophil histamine release could be differences in cell surface receptors for antigen-specific antibodies. Studies on human basophils and rodent mast cells demonstrated that signaling through the high affinity IgE receptor FcεRI is decreased by co-aggregating these receptors to the low-affinity IgG receptor FcγRIIb (Kepley et al. 2004). In addition, a study on human allergic patients has shown that even low levels of allergen specific IgG may have a blocking effect on the early phase skin reaction (Wittman et al. 1996). Furthermore, it was reported that stress-related hormone response after skin testing may be responsible for weak skin test reactivity in cats (Willemse et al. 1993).

In contrast to the ability of the FIT to correlate with IDT results, strong discordances were noted between the levels of allergen-specific, circulating IgE and the basophil sensitization, although we have applied the Fcε receptor alpha chain as detection system for circulating feline IgE. Thus, we should have monitored sensitizing IgE irrespective of the possibility that there might be non-sensitizing IgE allotypes in cats. Likewise, some studies on humans and dogs have demonstrated considerable variation in the correlation of allergen-specific serum IgE antibodies with allergen-induced immediate type reactions (Foster et al. 2003; Foster and O'Dair 1993; Laffort-Dassot et al. 2004; Purohit et al. 2005), whereas other studies on cats and dogs have shown significant agreement between serological and biological assays (McCall et al. 1997; Stedman et al. 2001). Since methods and reagents used in the various studies were different, it is difficult to compare the results of the various studies.

Even so, the question of whether antibody levels and effector cell sensitivities correlate remains a matter of controversy. Yet it is not unexpected that the results of a serological test did not correlate well with those of a biological assay, since both tests measure different components of an antibody response to antigen. A serum-based ELISA demonstrates circulating allergen-specific serum antibodies and misses cell-bound IgE while results of the

FIT and IDT mirror allergen-specific, sensitizing antibodies bound to basophils and mast cells. Moreover, data obtained from rodent and human mast cells have revealed that IgG can mediate the allergic reaction via IgG receptors (FcγR) (Kiekens et al. 2000; Tkaczyk et al. 2004). Therefore, IgG could cause a positive FIT or IDT response in view of a negative IgE-specific serological test.

Also, the fact that the antigens used in the FIT and IDT were a crude whole flea extract while the antigens used in the Allercept® test were recombinantly expressed components of flea saliva might be a reason for the difference between test results. Also in a study using defined purified and structurally folded allergen to investigate the relation between specific IgE, basophil degranulation, and skin sensitivity in human patients allergic to birch pollen strong discrepancies were reported between the magnitude of IgE levels and biological sensitivity (Purohit et al. 2005). However, when the same source of antigen has been used in both FIT and ELISA, the results were approximate at least at the end of a long and weekly repeated exposure period (week 28). This indicates that comparable results from serological tests for free antibodies and cellular sensitization with such antibodies may be obtained only when using identical antigen preparations plus at the “proper” time post-antigen exposure: At the end of 28 weeks of repeated flea exposure, there was an agreement between ELISA (free antibodies) and the FIT (basophil bound antibodies); however, not at all with IDT (mast cell bound antibodies). It may be feasible that some weeks later there might have been a significant correlation with IDT as well. This, however, is still to be tested.

Using the FIT on cats throughout experimental long-term flea exposure under controlled conditions, it could be shown that feline basophilic granulocytes provide a suitable tool for the detection of type I sensitization of cats against *C. felis*. Good agreement between FIT and IDT was observed. The FIT even appeared to become positive earlier than IDT and therefore be a more sensitive tool for detecting type I sensitization without bothering the animal with skin tests. Consequently, the FIT might provide a potential immunological supplement to the clinical diagnosis of flea bite hypersensitivity in cats by documentation of a qualitative and semi-quantitative sensitization of feline basophils to flea antigens. However, when it comes to diagnosing an allergy in the cat, FIT results always have to be interpreted in the light of the history, clinical signs, exposure to allergens, and elimination of differential diagnosis in the individual patient. In addition, further investigations are required regarding FIT results in cats with clear clinical signs of FAD.

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Herewith we declare that the experiments of this study comply with current German laws.

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