



## The immunopathogenesis of flea allergy dermatitis in dogs, an experimental study

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### Abstract

In this study, we investigated the development of clinical disease and immune responses in the development of an experimental model of flea allergy dermatitis. Dogs were randomly divided into four treatment groups and were infested with fleas on two different feeding schedules (continuous and episodic). Group 1 consisted of four non-exposed dogs (negative controls) and Group 2 consisted of six dogs exposed to fleas continually. Groups 3 and 4 consisted of 14 dogs each that were exposed to fleas on an episodic schedule (two consecutive days every other week for 12 weeks). Group 4 also received intraperitoneal injections of a low dose of lectin (ricin) with immunomodulatory properties. The purpose of Group 4 was to investigate the effects of ricin on enhancing the development of clinical signs, flea antigen-specific IgE levels and altering the number of CD4+ and CD8+ T cell subsets in peripheral blood. Clinical signs developed in all flea exposed dogs, however, the dermatology lesion scores were less and shorter in duration for continuously exposed dogs compared to episodic exposed dogs, independent of ricin treatment. Lesion development was concentrated in the flea triangle and consisted principally of erythema, followed by alopecia, excoriation, papules, and crusts. CD4+ and CD8+ lymphocyte subsets or IgE levels were not altered by ricin treatment. Flea antigen-specific IgE values were highest in dogs exposed to fleas on a continuous basis compared to those episodically exposed. A greater percentage of clinical responder dogs with negative flea-specific IgE titers or negative intradermal test (IDT) were present in the episodic exposure groups than in the continuous exposure group. IgE titers corresponded slightly better with clinical responders than the IDT. The agreement between the IgE titers and IDT was good (weighted  $\kappa = 0.67$ ). Histopathology of skin samples were consistent with a Type I hypersensitivity. In conclusion, we were able to develop a model of flea allergy dermatitis by experimentally exposing dogs to fleas on an episodic and continuous feeding schedule. In this study, continuously exposed dogs did not develop immunotolerance, and ricin did not enhance the development of FAD.

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### 1. Introduction

Flea bite hypersensitivity, also called flea allergy dermatitis (FAD) is the most common skin allergy encountered in small animal veterinary medicine, the

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immunopathogenesis of which has been little studied (Halliwell et al., 1987a; Halliwell and Schemmer, 1987b). Flea bite hypersensitivity manifests as pruritic dermatitis in animals that have become sensitized to antigenic material in flea saliva. Flea saliva contains a variety of histamine-like compounds, enzymes, polypeptides, and amino acids that span a wide range of sizes from 40 to 66 kDa (Halliwell et al., 1987a). They are also known to induce Type I, Type IV, and basophil hypersensitivity reactions. In general, most flea allergic dogs have immediate skin hypersensitivity (Gross and Halliwell, 1985). There are very few reports of experimentally induced models of flea allergic dermatitis in dogs (Gross and Halliwell, 1985; Halliwell, 1984a; Halliwell et al., 1987a; von Tscharner and Halliwell, 1990). In one study, five dogs were continuously exposed to fleas for 12 weeks and compared to two groups of five dogs exposed on an episodic basis. Dogs exposed on an episodic schedule developed positive intradermal tests and flea-specific IgE and IgG antibodies within 2–12 weeks, whereas the continuously exposed dogs developed allergic responses later and to a lesser degree (Halliwell, 1984a). Similar findings were noted in a later study in which groups of eight dogs intermittently exposed to fleas on a weekly schedule or three times weekly schedule developed positive skin tests within 3–8 weeks post-exposure, whereas continuously exposed dogs failed to develop positive skin tests. When the continuously exposed dogs were switched to an intermittent exposure at 24 weeks, they also developed skin test reactivity and antibody responses similar to the previous group of episodically exposed dogs (von Tscharner and Halliwell, 1990). In natural exposure settings, dogs that were continually exposed to high flea burdens or were completely flea naïve had low IgE and IgG antibody levels and negative intradermal tests compared to flea-hypersensitive dogs (Halliwell and Longino, 1985; von Tscharner and Halliwell, 1990). These observations suggest that dogs exposed on a continuous basis may become partially or completely immunotolerant and that this immunotolerance may be broken when the dogs are switched to an intermittent exposure.

The objectives of this investigation were to develop an experimental model of FAD in the dog and to evaluate the differences in the development of clinical signs, immune responses and lesions in dogs exposed

to fleas on a continuous versus an episodic feeding schedule. In an attempt to create an immunotolerant group, six dogs were exposed to fleas on a continuous feeding schedule. To create the FAD model we exposed 28 dogs to fleas on an episodic basis. Nanogram amounts of a lectin immunomodulator, ricin were administered to half of the episodic exposed dogs with the intent of boosting flea-specific IgE production and enhancing the development of clinical signs associated with flea exposure. This strategy was based on previous studies that linked inhalation of castor bean dust by mill and dock workers with increased incidence of allergic disease (Thorpe et al., 1989), and that administration of ricin with an antigen induced enhanced production of IgE in animals that were inherently low IgE responders (similar to non-atopic dogs or people) (Diaz-Sanchez and Kemeny, 1991). Ricin has been shown to enhance IgE responses by preferentially inhibiting a population of regulatory CD8+ T lymphocytes (Diaz-Sanchez et al., 1993) that tend to dampen IgE responses (Noble et al., 1993).

In this study, we used dermatological assessments, complete blood counts, CD4 and CD8 subset enumeration, flea antigen-specific IgE antibody responses, intradermal skin responses to flea antigens, and cytological and histological assessments of skin lesions to compare local and systemic responses to fleas in dogs exposed on continuous and episodic feeding schedules with or without concurrent ricin administration. We were able to identify differences in local and systemic responses among dogs developing FAD that were dependent on the feeding schedule, but independent of lectin exposure.

## 2. Materials and methods

### 2.1. Animals and housing

Female beagles were purchased from a Class A animal dealer and housed individually in cages or paired in runs. All dogs chosen for this study were greater than 1 year of age, because flea allergic dermatitis is rarely observed in dogs <12 months of age. While on study, all dogs were maintained under the guidelines of the Kansas State University Institutional Animal Care and Use Committee (IACUC).

## 2.2. Flea infestations

A total of 40 beagles were initially included in the study and divided into four groups. Group 1 consisted of six dogs that were non-flea exposed controls (two of these dogs were eventually removed from study due to complications in handling the dogs). A laboratory strain of *Ctenocephalides felis*, established and maintained on cats as a closed colony at Kansas State University since 1990 was used. Dogs were infested with fleas that were 1–3 days post-emergence. Six dogs (Group 2) were exposed to unfed *C. felis* on a continual basis by infesting each dog with 16 fleas on day 0, and 17 additional fleas every other day for 12 weeks (last infestation day 84). Total flea exposure during the study was 709 fleas. Group 2 dogs were expected to develop immune tolerance, as long as fleas were maintained on these dogs continuously throughout the study (Halliwell and Longino, 1985; Halliwell, 1984b; von Tscharnher and Halliwell, 1990). Verification of continuous flea exposure was determined by daily visual examination of the hair coat. Groups 1 and 2 dogs were housed in individual cage banks located on opposite sides of the room. Groups 3 and 4 dogs were pair-housed in 14 runs each containing one dog from each group. All dogs in Groups 3 and 4 were infested with 109 fleas on day 0 and then 100 fleas every other week for 12 weeks (709 total fleas). Following a 48 h infestation/exposure period, fleas were removed from Groups 3 and 4 animals by the oral administration of nitenpyram (Capstar: Novartis Animal Health). Thirty-six hours after treatments with nitenpyram dogs were visually examined and flea combed to verify that dogs were free of fleas. This provided a 12-day non-exposure period between each reinfestation (Dryden, 2002; Schenker, 2002).

## 2.3. Ricin administration

Group 4 dogs received intraperitoneal injections of ricin (500 ng in 0.5 ml of sterile saline) on day 0. Since none of the dogs showed a significant rise (two dilutions) in serum IgE titers by day 16, all were given a second injection of ricin on day 31. On day 42, nine dogs still had negative IgE titers and were given a third injection of ricin on day 56. All ricin injections were given immediately prior to flea infestations. (*Note:* Ricin is highly toxic and it was used strictly in

accordance with the regulations established by the CDC Select Agent Transfer Tracking System.)

## 2.4. Dermatological scores for clinical assessment

Blinded clinical dermatologic assessments were made on days –2, 2, and then bi-weekly for the duration of the study. A lesional scoring system was developed to quantitate erythema, papules, crusts, scale, alopecia, and excoriation. Each lesion was graded by a board-certified veterinary dermatologist using a scale from 0 to 3: 0, no signs; 1, mild; 2, moderate; 3, severe. Three body sites were assessed; (1) dorsum from the withers to the base of the tail; (2) right lateral thorax just caudal to the elbow and extending to the last rib; (3) “flea triangle” defined as the caudomedial thighs and ventral abdomen. The dermatology scores for each dog were recorded for each observation day in a table using the format depicted in Table 1. For the purpose of comparing the development of lesions between the treatment groups, each of the six dermatology categories for each body site were summed for each dog at each observation period and a grand total was computed (Table 1).

## 2.5. Peripheral blood counts and examinations

Complete blood counts were determined on days –2, 16, 30, 42, and 94 using a hematology analyzer (Cell Dyn 3700, Abbott Lake, IL). White blood cell populations were determined by manual differential counts. Buffy coats were evaluated for the presence of circulating mast cells. To prepare buffy coat smears a

Table 1  
Sample dermatology scoring report used for each observation period

	Site 1 (dorsum)	Site 2 (lateral thorax)	Site 3 (flea triangle)	Sum of all sites and categories
Erythema	1	1	2	4
Papules	0	0	2	2
Crusts	0	1	1	2
Scale	1	0	1	2
Alopecia	0	1	2	3
Excoriation	0	1	2	3
Total scores	2	4	10	16

Wintrobe tube (Becton Dickinson, Rutherford, NJ) was filled with 1 mL of whole blood (collected in K<sub>3</sub> EDTA anti-coagulant), then centrifuged at 1000 × g for 5 min, and the buffy layer was aspirated using a 9 in. Pasteur pipette. Blood smears and two buffy coat smears were prepared and stained using a modified Wright stain (Aogen, Kalamazoo, MI) and Hema-tek slide stainer (Bayer, Elkhart, ID).

## 2.6. Immunophenotyping of lymphocyte subsets

Lymphocyte subsets were determined (days –2, 16, 30, 42, 56, and 94) using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and commercially available monoclonal antibodies to CD4, CD8, and B lymphocytes (VMRD, Pullman, WA). The lymphocyte subsets were labeled using a whole blood assay previously described (Byrne et al., 2000). The percentage of each subset was multiplied by the absolute lymphocyte count to obtain the absolute numbers of CD4, CD8, and B lymphocytes.

## 2.7. Testing of IgE specific for flea salivary allergen

To monitor the rise in IgE antibody to flea salivary antigens, serum samples were collected on days –2, 16, 30, 42, and 94. A commercially available test (ALLERCEPT™, Heska, Fort Collins, CO) was used to monitor flea-specific IgE in serum samples that were collected on days –2, 16, 30, 42, and 94. Allerecept is a non-competitive, solid-phase enzyme immunoassay that incorporates a biotinylated Fc Receptor (FcεRIα) as the primary tracer for allergen-specific IgE molecules, streptavidin alkaline phosphatase as the enzyme containing secondary tracer, and *p*-nitrophenyl phosphate as substrate (Stedman et al., 2001). Plates were coated with a mixture of whole native flea saliva and a recombinant of the flea salivary antigen Ctef1. This assay recognizes serum antibodies that are heat labile and have been shown to be IgE; other antibody isotypes are not detected in this optimized assay. Results were expressed in ELISA absorbance (EA) units, which are milliabsorbance units that have been corrected for background and normalized to a five-point calibration curve. Such a normalization scheme removes the day-to-day variance that is inherent to any ELISA. The lower cut-off, based on the range

(mean ± 3 S.D.) of reactivity evident in negative samples and the susceptibility of a positive response to heat inactivation, was set at 150 EA units. A near linear response in the assay is evident across the detectable range of reactivity for which the lower detection limit is less than 100 pG/mL of specific IgE. Increase in response is directly proportional to the serum IgE content and is approximately equal to the square of the concentration factor. Thus, a two-fold increase in EA unit response is approximately equal to a four-fold rise in flea-specific IgE antibody content. To ensure that all responses throughout the duration of the study were detectable in the defined linear range of detection for the assay, varying dilutions (1:30, 1:90, and 1:270) of each sample were evaluated; however, only the results from the 1:30 dilutions are presented in this report.

## 2.8. Intradermal testing for reactivity to flea antigens

Intradermal tests (IDTs) were performed by established methods (Hillier and Deboer, 2001) on day 94 of the study. Each animal received a series of injections on the left ventral flank. Six injections of 10-fold serial dilutions of flea extract (stock solution 1:100 w/v, Greer Labs), as well as histamine (positive control, 1:100,000) and saline (negative control) were injected. Each injection was in a volume of 100 µl. The skin response was recorded as the diameter of the wheal reaction. Two measurements were taken, one perpendicular to the other and averaged. To help visualize the wheal reaction, each dog was injected intravenously with 5 ml of a 1.0% solution of sterile Evan's blue dye 5 min prior to skin testing. Skin responses were measured 15 min after injection and reactions were considered positive in each dog when the diameter of a wheal was greater than, or equal to, the average of the positive and negative control wheals. Delayed type hypersensitivity responses were not taken because it was not a focus of the study, plus the Evans blue dye administered intravenously prior to the intradermal skin test would have confounded the ability to assess these reactions post-IDT.

## 2.9. Cytological examination

Pustules of several dogs were aspirated over the course of the study and collected cells were examined

by light microscopy. Slides were stained with a differential stain (Protocol Hema 3, Fisher Scientific).

### 2.10. Histopathologic examination

Punch biopsies (6 mm) were collected from the dorsum and lateral thorax region of each dog on days –2 and 94 of the study. The samples were preserved in neutral buffered formalin for histological analysis. Numbers of mast cells were evaluated in each section using a Giemsa stain. Total numbers of Giemsa positive cells were recorded separately for each site. Histologic changes were scored with the following grading system—0: no changes; 1: superficial dermal edema; 2: superficial dermal infiltrates of mast cells, eosinophils and mononuclear cells; 3: dermal edema and cellular infiltrates; 4: infiltrates heavily concentrated around vessels and adnexa; 5: epidermal pustules and/or suppurative folliculitis; 6: dermal fibrosis with or without infiltrates.

### 2.11. Statistical analysis

Analysis of variance (ANOVA) for repeated measures was used to determine differences among treatment groups, days of infestation, and the interaction between treatment and days of infestation. The data was log transformed. Level of significance was considered  $P < 0.05$ . Differences in IgE (1:30 dilution) were compared by analysis of variance for repeated measures with day –2 results included as a covariate. A frequency analysis was used to determine the differences in the development of various lesion categories among treatment groups. A frequency analysis was used to compare histopathologic changes among treatment groups.

Using Group 1 as the animals without FAD, a cut-off value for dermatology scores was determined as 3 standard deviations (S.D.) above the mean of the flea triangle scores (measured at day 56 of the study). This cut-off value (dermatology score  $>2.0$ ) was used to classify animals in Groups 2–4 as clinical responders or clinical non-responders. A weighted kappa ( $\kappa$ ) statistic was performed on the data to determine the amount of agreement between the intradermal test and the Allercept IgE test in the experimental dogs with FAD (Altman, 1991) using Analyse-it Software Ltd., version 1.65 (Microsoft Excel).

## 3. Results

### 3.1. Clinical observations

Dermatology lesions were first noted by the second day of flea exposure in the episodically exposed groups, whereas dogs exposed on a continuous basis did not show lesions until day 16 (Fig. 1a). The dermatology scores reached peak mean values in Groups 2 and 4 by day 56, scores in Group 3 dogs increased until day 88. There was no difference in the scores between all flea exposure groups at day 56 (Fig. 1a and Table 2). By day 74, the continuously exposed dogs showed a decline in dermatology scores compared to the episodic groups. The dermatology scores decreased by 50% of peak scores in both groups of episodically exposed dogs at day 98 of the study, 14 days after the last flea exposure (Fig. 1a and b).

The flea triangle contributed to the largest proportion of the dermatology scores in all flea exposed groups (Fig. 1b and Table 2). The dorsum and lateral thorax regions contributed minimally to the dermatology scores of flea exposed dogs, but they contributed to the majority of the scores for the negative control group (data not shown). Erythema was the major component of the lesion development in all flea exposed dogs compared to negative controls ( $P < 0.001$ ), with over 50% of the dogs in Groups 3 and 4 developing mild or moderate erythema scores by day 2, whereas over 80% of Group 2 dogs did not develop mild erythema until day 16 (Fig. 2). Moderate erythema was maintained in over 60% of all flea exposed dogs between days 56 and day 88; after which the clinical signs diminished (Fig. 2). The sequence of lesion development was as expected with erythema occurring first, followed by alopecia, excoriation, crusts and pustules. Alopecia was significantly different from negative controls at day 44 for Group 2 and at day 56 for Group 3 dogs. Excoriation peaked at day 72, however, only Group 3 dogs had a frequency of reactor dogs with lesions scores significantly different from negative controls ( $P = 0.010$ ). All flea exposed groups had the highest frequency of dogs with pustules at day 88 of the study compared to controls ( $P < 0.05$ ). Group 2 had the highest frequency of dogs with crusts at days 88 ( $P = 0.03$ ) and 94 ( $P = 0.04$ ) compared to controls and Groups 3 and 4.

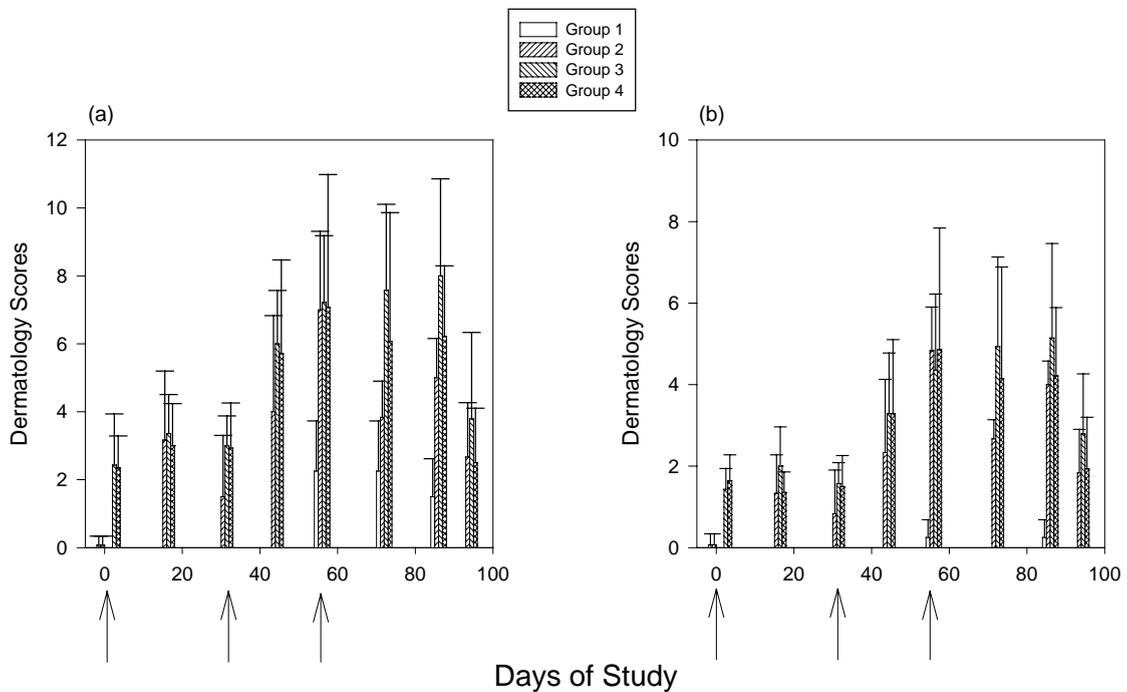


Fig. 1. The development of clinical signs during the course of the study is recorded for each treatment group. The total sum of the dermatology scores for all three body sites are plotted (mean  $\pm$  1 S.D.) (a), and scores for the flea triangle region are plotted in (b). Arrows indicate administration of ricin.

There were no significant differences noted between treatment groups for scale at this site.

On the dorsum and lateral thorax, erythema was the only clinical sign which the flea exposed groups developed that was greater than the negative controls. At the dorsum, only Group 2 and 3 dogs showed erythema at day 44 that was greater than controls ( $P < 0.05$ ). At the lateral thorax site, all flea exposed groups had significantly higher proportions of dogs

with erythema than controls between 30 and 88 days of the study ( $P = 0.01$ ).

### 3.2. Peripheral blood evaluation

No clinically significant drop in hematocrits was noted for any of the flea exposed dogs, nor did the values for any of the dogs drop below the reference interval for the laboratory (37–55%). There were no

Table 2  
Summary of flea allergic responses in treatment groups

Treatment group	Highest total clinical score at day 56 (mean $\pm$ 1 S.D.)	Highest mean clinical score for flea triangle at day 56 for (mean $\pm$ 1 S.D.)	IgE (>150 units), number positive dogs/total (low–high interval)	Intradermal test number positive dogs/total (mean titer <sup>a</sup> )
1 (non-exposed)	2.3 $\pm$ 1.5	0.25 $\pm$ 0.4	1/4 (0–584)	0/4
2 (continuous)	7.0 $\pm$ 2.3	4.8 $\pm$ 1.1	6/6 (291–3510)	5/6 (5 dogs with $10^3$ )
3 (episodic)	7.2 $\pm$ 2.0	4.4 $\pm$ 1.9	12/14 (0–4072)	10/14 (9 dogs with $10^3$ ; 1 dog with $10^4$ )
4 (episodic + ricin)	7.1 $\pm$ 3.9	4.9 $\pm$ 2.9	8/14 (7–2159)	11/14 (9 dogs with $10^3$ ; 1 dog with $10^4$ ; 1 dog with $10^6$ )

<sup>a</sup> The titer is defined as the reciprocal of the highest dilution that produced a positive result.

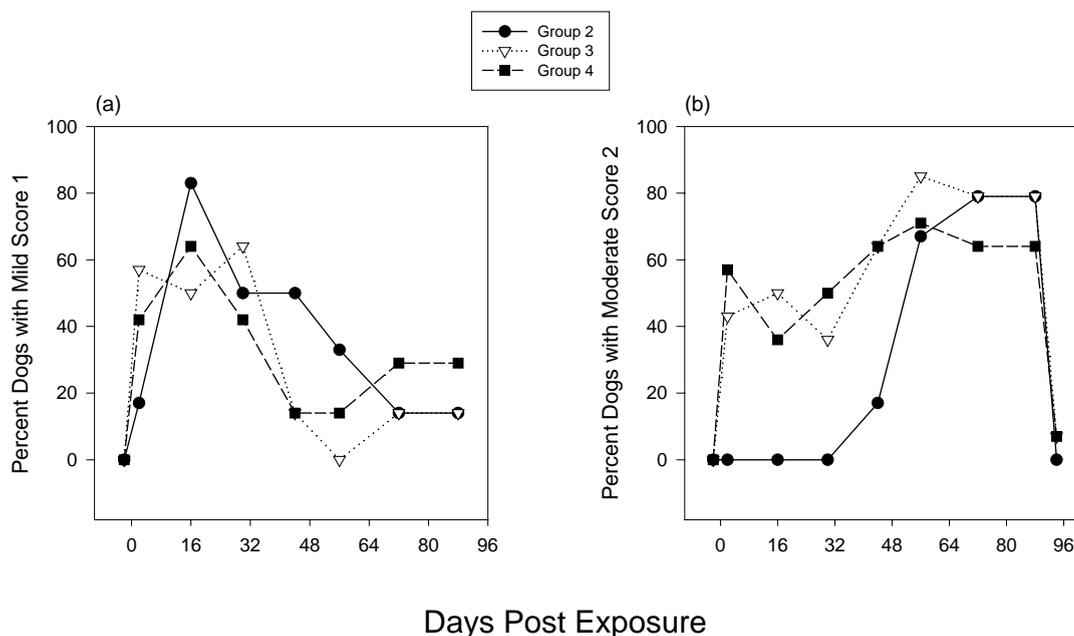


Fig. 2. The proportion of dogs that developed erythema scored as mild (score 1) (a) or moderate (score 2) (b) over the course of flea infestation is plotted for Groups 2 (circle), 3 (triangle), and 4 (square).

significant differences in the numbers of circulating basophils, eosinophils, or total number of lymphocytes noted during the course of this study (data not shown). The mean number of basophils, eosinophils, and lymphocytes for all groups throughout the course of the study ranged between 0–42, 100–600 and 1300–2800 per microliter, respectively. Basophils and eosinophil concentrations were within laboratory reference intervals (0–100 and 0–900 per microliter), but the lymphocyte concentrations occasionally dropped below the reference intervals (2000–7000 per microliter). No mast cells were identified in any of the buffy coat preparations.

### 3.3. Lymphocyte subsets

The proportion of CD4+, CD8+, and B lymphocytes determined prior to flea infestation was similar to published values for other groups of beagles (Byrne et al., 2000; Faldyna et al., 2001). The numbers of B lymphocytes defined by CD21 did not change during the course of the study. There were no differences in the numbers of circulating lymphocyte subsets (CD4 and CD8) between treatment groups for the duration of the study (data not shown).

### 3.4. Flea allergen-specific IgE

Dogs on the continuous flea exposure schedule had higher IgE values (1:30 dilution of serum was the optimal dilution) compared to groups exposed on an episodic basis (Fig. 3,  $P < 0.05$ ). This was reflected in the observation that the IgE levels rose earlier during the course of flea infestation in continuously exposed dogs compared to the episodically exposed dogs (Fig. 3). Flea antigen-specific IgE levels (mean titers) in continuously exposed dogs was significantly above control dogs on day 32, whereas IgE levels in episodic exposed dogs did not rise above control values until day 42 or later (Fig. 4). At the conclusion of the study, the mean IgE values for flea exposed groups were significantly higher than controls ( $P < 0.05$ ). However, there were no significant differences in the final titers between flea exposed groups. All dogs in the continuous exposure group had IgE values above the cut-off (>150 EA units) including four dogs with high values >1500 EA units (Table 2).

Development of flea-specific IgE titers correlated well with the development of clinical signs in the continuous exposure group; all dogs reacted (Table 3).

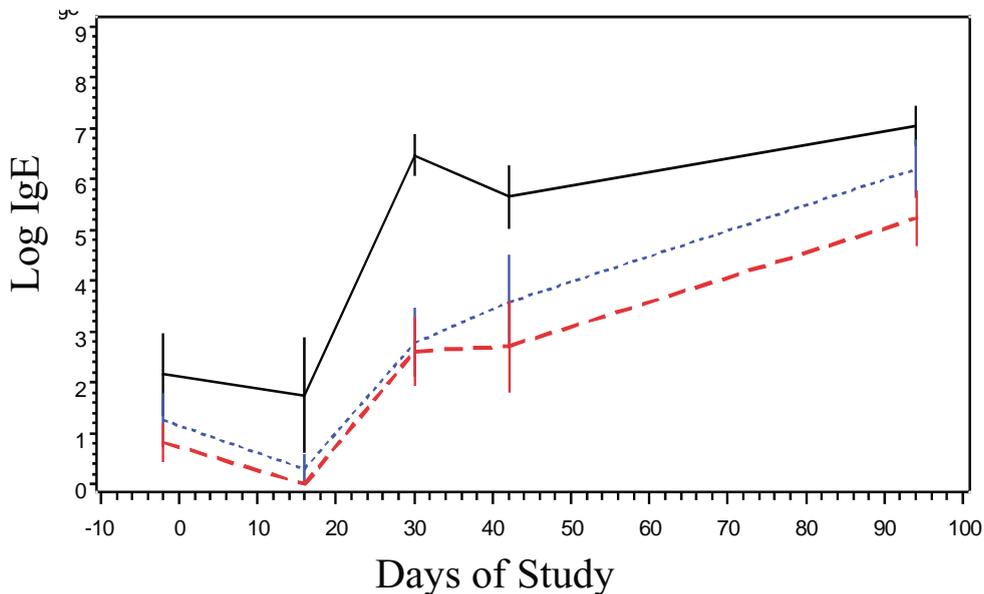


Fig. 3. Log transformed results of IgE concentrations ( $\ln(\text{IgE} + 1)$ ) for flea exposed dogs indicate that dogs exposed to fleas on a continuous schedule (Group 2, solid line) had higher IgE than dogs exposed to fleas episodically without ricin (Group 3, short dashed line) or with ricin treatment (Group 4, long dashed line).

In contrast, 18 of 28 (64%) dogs exposed to fleas on an episodic feeding schedule (10 in Group 3 and eight in Group 4) had IgE titers above the cut-off that corresponded with the development of significant clinical responses ( $\geq 3$  S.D. of the mean flea triangle scores for Group 1) (Table 3). Five dogs (two in Group 3 and three in Group 4) did not develop significant IgE titers even though they showed clinical responses to fleas. Three dogs in Group 4 that had insignificant IgE titers failed to develop clinical signs above that of non-exposed dogs. On the other hand, there were two clinically non-responder dogs in Group 3 and one in Group 1 with flea-specific IgE values above the EA cut-off. Three control dogs had flea-specific IgE values below the cut-off throughout the study, whereas one had an EA value of 584 at the end of the study.

### 3.5. Intradermal test

Five out of six dogs in the continuous feeding group had positive intradermal tests, whereas none of the animals in the control group tested positive (Table 2). Ten out of 14 dogs tested positive in Group 3 and 11 dogs were positive in Group 4. The highest dilution of flea antigen at which the IDT gave a positive reaction

in most dogs was  $10^3$  (Table 2). One dog each in Groups 3 and 4 reacted at the  $10^4$  dilutions, whereas a single dog in Group 4 had wheal reactions out to a dilution of  $10^6$  of flea antigen.

Twenty-three of the 26 flea exposed dogs had positive IDT and significant dermatology scores

Table 3

Summary of flea-specific IgE and IDT responses based on clinical dermatology scores

	Flea-IgE test ( $>150$ EA units)	Flea-IgE test ( $<150$ EA units)	IDT positive test	IDT negative test
Derm scores $> 2.0$ (3 S.D.)				
Group 1	0	0	0	0
Group 2	6	0	5	1
Group 3	10	2	9	3
Group 4	8	3	9	2
Total	24	5	23	6
Derm scores $\leq 2.0$ (3 S.D.)				
Group 1	1	3	0	4
Group 2	0	0	0	0
Group 3	2	0	1	1
Group 4	0	3	2	1
Total	3	6	3	6

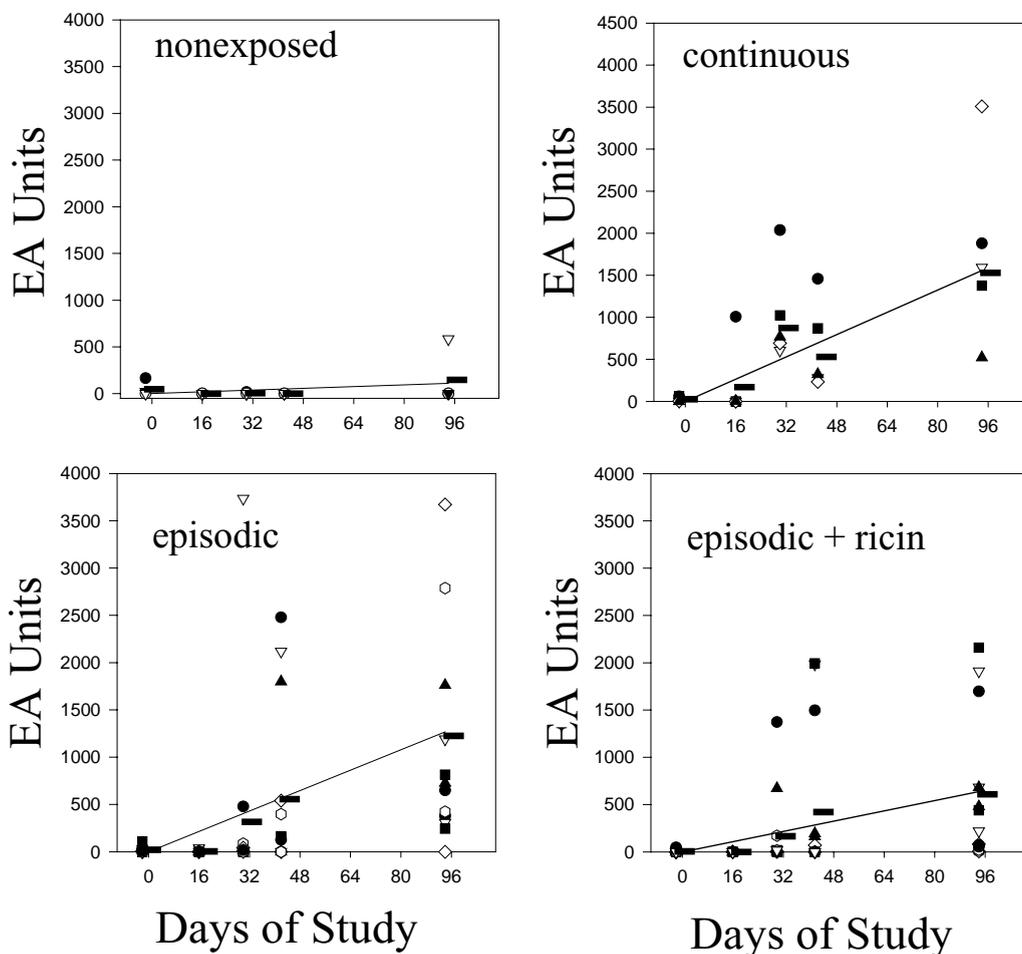


Fig. 4. Flea antigen-specific IgE concentrations recorded during the course of flea infestation for individual dogs of each group. Each symbol represents a value for a dog. The thick horizontal bar represents the average of the group. The line is the best fit line for the data.

representative of FAD (Table 3), five dogs from the continuously exposed group and 18 from episodic exposed group. There were three dogs (one in Group 3 and two in Group 4) that were IDT positive but were classified as clinical non-responders. Six dogs (one in Group 2, three in Group 3, and two in Group 4) had significant dermatological lesions without positive IDT. One dog each in Group 3 and in Group 4 was clinically non-responsive and negative for IDT.

When the combination of positive IDT and IgE titers ( $>150$  EA units) in dogs with clinical scores of  $>2.0$  were considered a total of 18 dogs in the flea exposed groups fit this criteria (Table 4) including

five of the six in the continuously exposed group, seven of 14 in Group 3, and six of 14 in Group 4. One dog in the continuously exposed group was negative by intradermal skin test, but had an IgE value of 522 and a clinical score of 5. Similarly, six flea exposed dogs (one in Group 2, three in Group 3, and two in Group 4) tested negative by IDT but had high IgE levels and clinical scores  $>2.0$  (Table 4). One dog in Group 3 with a negative IDT and low clinical score had a high IgE value, whereas another clinical non-responder dog in Group 4 did not produce significant IgE values nor reacted by IDT. Two dogs in Group 4 with negative IgE and clinical scores  $<2.0$  had a positive IDT. Fig. 5 illustrates a

Table 4  
Weighted  $\kappa$ -test for agreement between IDT and Allercept IgE

Intradermal skin test	Flea-specific IgE				Total
	Positive test/CR	Negative test/CR	Positive test/NCR	Negative test/NCR	
Positive test/CR	<b>18</b>	4	0	0	22
Negative test/CR	6	<b>1</b>	0	0	7
Positive test/NCR	0	0	<b>1</b>	2	3
Negative test/NCR	0	0	2	<b>4</b>	6
Total	24	5	3	6	38

The amount of agreement is based on the proportion of cases concentrated on the leading diagonal (numbers in bold). Positive test: IgE > 150 EA units; negative test: IgE < 150 units; CR: clinical response; NCR: no clinical response.

dog with a positive IDT ( $10^3$ ) and a dog with a negative IDT. The amount of agreement between the IDT and Allercept IgE test was good (weighted  $\kappa$ -value of 0.67, Table 4) or 67% greater than that expected by chance alone (Altman, 1991). Complete agreement occurs when the data is concentrated on the leading diagonal (Table 4, numbers in bold). In this study, 24 of the 38 dogs that had complete agreement (Table 4). There were more clinically responsive dogs with negative IDT results compared to the IgE test and equal numbers of dogs with positive results for the IDT and IgE test that were non-clinical responders.

### 3.6. Cytologic examination

Isolated pustules developed in four of five dogs in Group 2, seven of 14 dogs for Group 3, and seven of 14 in Group 4. The fine needle aspirate preparations from these lesions consisted of neutrophils often with intracellular cocci and rod shaped bacteria.

### 3.7. Histopathologic examination

The numbers of Giemsa positive cells in skin biopsies taken from the dorsum and lateral thorax were compared between treatment groups collected



Fig. 5. A typical wheal response to histamine (lower wheal) and flea antigen (upper wheal) in a responder dog (a) at a 1:10 dilution of antigen and non-responder dog (b). Saline control injection site is to the right of the histamine injection. Dilutions of flea antigen in the top row from left to right are as follows ( $10^3$ ,  $10^4$ ,  $10^5$ ,  $10^6$ ,  $10^7$  and  $10^8$ ).

at the beginning (day –2) and end of the study (day 94). The Giemsa stain highlighted the granules in mast cells. There were no significant differences in the number of Giemsa positive cells among the treatment groups at either biopsy site or between sampling dates. Common histological changes consisted of mild to moderate superficial edema and perivascular dermatitis with a mixture of mast cells, eosinophils, and mononuclear cells. The epithelium was not hyperplastic, but had mild parakeratosis. Some dogs had suppurative folliculitis and epidermal microabscesses and occasional dermal fibrosis. Only dogs in the continuously exposed group had skin lesions of significance in the dorsum samples.

Using the histology scoring system, the lesions in the thoracic biopsy samples of all treatment groups had significantly higher scores than the unexposed group ( $P < 0.01$ ). Moreover, dogs exposed on a continuous feeding schedule had the highest mean score ( $10.6 \pm 5.1$ ) compared to dogs in Group 3 ( $7.2 \pm 2.3$ ) and dogs in Group 4 ( $6.5 \pm 3.9$ ). For the dorsum biopsy sample, only dogs in the continuous exposed group were significantly different from unexposed controls.

#### 4. Discussion

In this report, clinical FAD was reproduced in dogs exposed to fleas on either a continuous or episodic basis. This is the first study to our knowledge that documents the temporal development of the dermatological lesions in an experimental model of FAD using a detailed categorical scoring system. A similar scoring system has been used in naturally affected dogs to assess immunotherapy (Kwochka et al., 1998). The flea triangle region contributed to the majority of the dermatology lesion scores for all flea exposed dogs. Erythema developed sooner and persisted longer in the dogs exposed episodically as compared to those continuously exposed. The delayed appearance of erythema in the continuous group can be explained by the fact the total flea burden for these dogs was not equal to the episodic group until day 12 in which they received the same amount of fleas as the episodic dogs received on the first day. Although the delayed and lesser clinical response in the continuously exposed dogs is consistent with previous studies (Halliwell,

1984a; von Tscharner and Halliwell, 1990), the dogs in our study did not develop immunotolerance but developed clinical signs of FAD, had allergen-specific IgE responses, and reacted to intradermal injection of whole flea extract. Although flea-specific IgE responses were higher than those exposed on an episodic basis, the lesion scores in the continuously exposed group were less than those of the episodic groups. We noted that one dog in the continuously exposed group was non-responsive by IDT and two animals had relatively low IgE titers compared to others that responded (291 and 522 versus 1377–3510, respectively). It is possible that a longer period of continuous exposure or higher flea burden might have induced immunotolerance in these animals. Contrasting the results of the current study with that of prior work is difficult because the specific number of fleas used to infest continuous exposed dogs were not specified (Halliwell, 1984a; von Tscharner and Halliwell, 1990). However, we speculate that perhaps the slow build up of flea antigen exposure that occur in our study contributed to the development of hypersensitivity in the continuously exposed group compared to dogs in natural environments that are exposed initially to heavy flea burdens. For intermittently exposed animals, a group of dogs exposed to 25 fleas for 15 min once weekly and a group exposed to 25 fleas three times weekly (75 fleas) developed positive immediate and delayed skin tests by 3 and 8 weeks, respectively (von Tscharner and Halliwell, 1990). Although flea burdens between our study and the group exposed to 75 fleas weekly in the von Tscharner and Halliwell (1990) would be similar, comparisons are difficult because the clinical assessments varied (IDT for early studies and dermatology scores for our study). The reason we maintained fleas on dogs for 2 days in our study is that we have conducted numerous flea product efficacy evaluations where flea removal was conducted 48 h after reinfestations. In several of those studies, we observed dogs developing clinical signs consistent with FAD after several weekly reinfestations (unpublished work). The current study was designed to mimic what we had observed previously.

As has been previously published, flea-specific IgE titers did not correlate well with severity of clinical disease in flea allergic dogs. In dogs that had IgE titers but no clinical signs, we propose that more than just elevated IgE is relevant to expression of clinical disease.

In other words, the dogs were hypersensitive to flea saliva producing flea-specific IgE, but did not develop clinical disease when exposed to fleas. This indicates that IgE is necessary but not sufficient for development of clinical disease. For example, dogs are likely to differ in the ease with which mast cells degranulate, or in the release of mast cell enzymes that regulate degranulation and therefore provides a different pruritic threshold (Mellon et al., 2002; Edston et al., 1999). Dogs that reacted clinically but showed no rise in IgE values above the cut-off during the sampling period may be explained by the fact that IgE has a short half life (2–3 days) (Tizard, 2000) or the possibility that IgE levels in episodically exposed dogs fluctuate more than in the continuous flea exposed group. Alternatively, IgE levels in the serum of these dogs may be relatively unchanged because of sequestration on tissue mast cells mobilized to the site of flea allergen exposure. Previous observations in early immunoassays using polyclonal or monoclonal antibodies to IgE noted that detection of IgE is impaired by the interference of canine IgG autoantibodies that complex with IgE (Hammerberg et al., 1997). Although an advantage to the Allercept assay is that the biotinylated IgE Fc receptor reacts specifically with IgE and not to purified canine IgG, the possibility that this assay may react to a heat stable form of a IgG subclass (IgGd) recently described in atopic dogs has not been ruled out. However, the possibility that allergen-specific IgG might be obscuring specific IgE measurements in the Allercept assay has not been supported by experimental results that demonstrate a large excess of purified IgG (specific and/or non-specific) does not interfere with IgE detection (Stedman et al., 2001). Further, serial dilution of allergen-specific IgE into sera containing extremely high levels of specific IgG yields results that are indistinguishable from results that are observed when the specific IgE is diluted in buffer alone (personal communication with Kenneth W. Lee). The reason why the dog in Group 1 developed a positive IgE titer at the end of the study is not known; however, it may represent inadvertent exposure to fleas since the non-exposed group of dogs was housed in the same room as the continuously exposed group.

Mast cells were not identified in buffy coat smears of any of the flea exposed dogs of this investigation. In a study by Cayatte et al. (1995), six of 26 client owned dogs with naturally occurring flea bite hypersensitivity

had low numbers of circulating mast cells in buffy coat smears (Cayatte et al., 1995). The reason for the difference between these studies is not clear, however, the methods of buffy coat preparation differed and the published report indicated that most of the dogs had secondary pyoderma. The severity or extent of the pyoderma in that report was not described.

We did not observe any changes in circulating CD8+ T lymphocyte subsets in the group of dogs treated with ricin. Rats immunized with bee venom phospholipase A2 and ricin showed a dramatic increase in the CD4/CD8 ratio due to a 40% decrease in CD8+ T lymphocytes occurring between days 7 and 21 after immunization (Diaz-Sanchez et al., 1993). Compared to CD4+ cells, this population of regulatory CD8+ T lymphocytes had high affinity receptors for the ricin lectin (Diaz-Sanchez et al., 1993). It is hypothesized that the lectin enters the activated cell and inhibits cellular protein synthesis resulting in killing of the cell (Diaz-Sanchez and Kemeny, 1990). We did not observe a decrease in CD8+ lymphocytes in dogs treated with ricin, nor did this group develop higher IgE responses than dogs exposed on a continuous or episodic feeding schedule without ricin. Further studies are needed to determine if ricin affects canine CD8 T cell subsets as described in studies performed on the rat. Since ricin is a potent toxin with special handling requirements, it would be of interest to investigate the use of other immunomodulators in future studies, such as heat killed *Bordetella pertussis*, a known adjuvant which induces strong Th2 responses and IgE production in animals (Yilmaz et al., 1996; Sekiya, 1983; Hall et al., 1982; Clausen et al., 1969).

The skin lesions in flea exposed dogs were consistent with those described in earlier reports of experimentally induced flea bite hypersensitivity (Gross and Halliwell, 1985). The primary lesion was a superficial perivascular dermatitis and edema with mast cells, eosinophils, and mononuclear cells. These lesions were consistent with a Type I hypersensitivity reaction and not delayed hypersensitivity. Other than mild hyperkeratosis epidermal hyperplasia was not evident in the biopsy sites. The mild nature of the lesions was attributed to the biopsy site (thoracic and dorsum sites) which had less severe dermatologic scores than the flea triangle region. While DTH may be the only response noted in some flea exposed dogs, the incidence is low (von Tscharner and Halliwell, 1990;

Reedy et al., 2003). Since the focus of this study was to develop a model for the induction of clinical FAD in which several immune system parameters and the temporal progression of skin lesions were monitored, DTH was not our main interest and will have to be examined in this model at a later date. However, since IgE has been reported to induce delayed hypersensitivity responses via signaling through FcεR1 engagement on antigen presenting cells (Kraft et al., 2001; Ptak et al., 1991), it is reasonable to assume that dogs with flea-specific IgE, even those with low IgE titers, have an increased risk for IgE mediated DTH.

The proportion of dogs with FAD in this study that had positive IgE titers and IDT was just over fifty percent (18/34). Based on clinical scoring, there were two non-responder dogs in Group 3 and three in Group 4. Interestingly, the dogs treated with ricin had the highest number of dogs that were non-responders based on one or more criteria including low dermatology scores, low IgE values, and negative IDT. In contrast to a study that compared an allergen-specific antibody test to the IDT (Codner and Lessard, 1993), we showed better agreement between the IDT and Allercept IgE test (weighted  $\kappa$ -test = 67%). The reason for this observation may have been attributed to that fact that we used the weighted  $\kappa$  statistic instead of the  $\kappa$  statistic. The former test examines the amount of disagreement and agreement between the two assays (Altman, 1991). Although a positive IDT is not always indicative of allergy, it could indicate a sub-clinical hypersensitivity state, this test is considered a valuable tool if standardized methods provided by American College of Veterinary Dermatology are followed (Hillier and Deboer, 2001).

This experimental model of FAD produced a milder form of the clinical syndrome than other reports with naturally occurring FAD. The principal lesions in this study were erythema and alopecia. None of the dogs developed lesions compatible with hot spots or pyotraumatic dermatitis. This observation may be explained by the infestation dose of fleas, the duration of exposure, and/or the breed of dog used in the study.

## 5. Conclusion

We established an experimental model of FAD in the dog and showed that in this model dogs exposed to fleas

on a continuous feeding schedule can develop FAD and that immunomodulation with low doses of ricin did not accelerate the production of allergen-specific IgE.

## 6. Addendum

Since the completion of this study, 20 dogs (10 dogs each from Groups 3 and 4) were selected for a second study. After a 2-week period of rest, the dogs were re-exposed to 100 fleas on an episodic basis (every 2 weeks) for two additional months. By the end of the second month of re-exposure, many of the dogs began to show classic signs of flea bite hypersensitivity including extensive areas of erythema, papules, excoriation and pyotraumatic dermatitis near the tail head, indicating a 20-week, rather than a twelve week, exposure period may be more successful in inducing the classical signs of FAD.

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