

Characterization of allergens of the cat flea, *Ctenocephalides felis*: detection and frequency of IgE antibodies in canine sera

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SUMMARY

Flea allergens, fractionated by polyacrylamide gel electrophoresis and transferred to nitrocellulose, were identified using 20 flea-allergic dog sera in an enhanced chemiluminescent assay for canine IgE antibodies. At least 15 different flea components in the molecular weight range of 14–150 K bound IgE and every serum demonstrated a different pattern of binding. Three of the components with apparent molecular weights of 25, 40 and 58 K were each bound by at least 40% of the sera. No reactivity was seen when normal dog sera were used. These results demonstrate a greater number of flea allergens and a far greater diversity of the IgE antibody response to flea allergens than has previously been described, and suggest that immediate hypersensitivity may be an important mechanism in the pathogenesis of canine flea allergy.

Keywords flea allergy dermatitis, *Ctenocephalides felis*, allergen, immediate hypersensitivity

INTRODUCTION

Allergy to bites of fleas belonging to the genus *Ctenocephalides* may afflict up to 40% of the canine population and is the most common dermatological disorder encountered in veterinary practice (Nesbitt & Schmitz 1978, Keiffer & Kristensen 1979, Halliwell, Preston & Nesbitt 1987). Flea allergy dermatitis (FAD) is the term used to describe the full clinical syndrome which involves pruritis, the cardinal feature, and the typical lumbo-sacral distribution of chronic skin lesions (reviewed by Scheidt 1988).

Previous work has demonstrated that the oral secretions of the flea contain the components responsible for producing the hypersensitivity reactions of FAD (Benjamini, Feingold & Kartman 1960). Despite this, few studies designed to identify flea allergens have been reported, and little data is available on the relative clinical importance of individual flea allergens. In addition, the issue of whether the allergic reaction which occurs following a flea bite, results from an IgE-mediated reaction or one that is delayed, is still controversial (Benjamini, Feingold & Kartman 1961, Gross & Halliwell 1985).

With the availability of the immunoblotting technique which has been successfully used to detect and characterize IgE binding proteins from a range of allergen sources (Tovey & Baldo 1987), we have been able to identify at least 15 allergens in a whole body extract of the cat flea, *Ctenocephalides felis*. The binding pattern of flea-specific IgE antibodies was examined using the sera of 20 flea-allergic dogs in order to determine which flea components are likely to be important allergens and to determine the frequency of the IgE response to these components.

MATERIALS AND METHODS

Canine sera

Serum samples were obtained from 30 dogs where flea allergy dermatitis was suggested by one or more of the

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following criteria: positive intradermal skin test to Greer flea antigen (Greer Laboratories, Lenoir, NC), clinical history of reactivity to fleas and/or pruritis and chronic lumbo-sacral skin lesions.

Normal sera were collected from 5 dogs with a negative intradermal skin test to Greer flea antigen. Bulk canine sera were obtained from euthanased dogs and then screened for high IgE levels using a previously available anti-canine IgE (Calbiochem, San Diego, CA). Sera that had the highest levels of IgE were pooled for fractionation.

Rufus (Ruf) serum was taken from a dog with a 5+ positive intradermal skin test (see below) to Greer flea antigen.

Fractionation of canine sera

The IgE rich fraction from the bulked canine sera were obtained according to the method described by Halliwell, Schwartzman & Rockey (1972) with the following modifications. The DEAE-cellulose excluded fraction was freeze-dried and then dialysed against 0.2M NaCl, 2mM EDTA and 10 mM NaPO₄ (pH 8.0) before further fractionation on a 2.6 × 100 cm Sephacryl S-200HR column (Pharmacia, Uppsala, Sweden), using a 0.2 M NaCl, 2 mM EDTA and 10 mM NaPO₄ (pH 8.0) buffer. The proteins eluting prior to and in the first shoulder of the IgG peak (identified by dot blotting the fractions) were pooled, dialysed and freeze-dried.

Antisera to canine IgE

The IgE rich fraction was used to immunize rabbits. The immunization schedule was as previously described (Halliwell *et al.* 1972) with the following modification, after the third immunization rabbits were boosted with 500 µg of human myeloma IgE (Chemicon International, Temecula, CA). The antisera were made specific for canine IgE by repeated passage through a Sepharose 4B column (Pharmacia) coupled with a canine serum low in IgE that had been heat inactivated at 56°C for 4 h. The IgG fraction was then purified using Protein A sepharose (Pharmacia).

Intradermal skin testing

Dogs were sedated with 1.0 mg/kg of xylazine (Bayer Ltd., Botany, NSW) and atropine sulphate (Apex Laboratories, St Mary's, NSW) and injected intradermally on the lateral portion of the thorax with 0.1 ml of the test solution. A negative control of sterile saline and a positive control of histamine 1:100 000 w/v (Sigma, St Louis, MO) were always used. After 15–20 min, the weals were

arbitrarily scored—the negative control being 0 and any larger weal was given a positive value from 1+ to 5+, with 5+ being the most intense reaction.

Reversed cutaneous anaphylaxis

Two normal dogs were intradermally injected with ten-fold dilutions (in sterile saline) of the rabbit anti-canine IgE. Normal rabbit serum and rabbit anti-dog IgG (Sigma) were also used as controls. Weals were scored after 15 min.

Prausnitz-Kustner (PK) neutralization tests

An equal volume of Ruf serum and either the anti-canine IgE or normal rabbit serum were incubated for 2 h at room temperature on a rotary suspension mixer. After standing overnight at 4°C, the samples were centrifuged (5000 g, 8 min), and doubling dilutions made of the supernatant in sterile saline. A flea skin test-negative dog was then intradermally injected with 0.2 ml of each dilution. After 24 h, the sites were challenged with 0.1 ml of Greer flea antigen (1:1000).

Flea extract for SDS-PAGE

Fleas were obtained from experimentally infected cats in our laboratory according to established procedures (Woodley 1980) and from Ciba-Geigy Ltd (Kemps Creek, NSW).

Whole frozen fleas were weighed and ground with a mortar and pestle on ice. SDS was then added to a final concentration of 5% (w/v). The slurry was then centrifuged (10 000 g, 5 min) and the supernatant mixed 1:1 with a reducing sample buffer, aliquotted and frozen.

Gel electrophoresis

One ml of flea extract containing 2 mg of protein was run on a 10% discontinuous SDS-PAGE preparative gel as previously described (Laemmli 1970), using a Protean II cell (BioRad, Richmond, CA) at 40 mA/gel for 2–3 h.

After electrophoresis, 2 outer edges of the gel were stained with 0.025% (w/v) Coomassie blue R250 (Sigma) in 30% methanol and 10% glacial acetic acid. Low range SDS-PAGE molecular weight standards (BioRad) were run along with the flea extract on a separate gel and stained with Coomassie blue.

Western blotting

Proteins were electrophoretically transferred at 5°C to Hybond-C super (Amersham, Amersham, UK) in a

transblot cell (BioRad) using a 25 mM NaPO₄ pH 6.5 buffer, overnight at 25 °C.

After transfer, the membrane was blocked with PBS containing 0.5% (v/v) Tween 20 (BDH, Poole, England), at room temperature for 30 min. The membrane was then cut into 0.5 cm strips and incubated overnight at 4 °C on a rocking platform with 500 µl of canine serum diluted 1:1 with PBS Tween.

After five 5 min washes with PBS-Tween, the strips were incubated with rabbit anti-canine IgE at 1/3000 in PBS-Tween overnight at 4 °C. The strips were then washed again and incubated with Donkey anti-rabbit IgG-HRP labelled antibody (Amersham) at 1/3000. Immunoblots were developed using the enhanced chemiluminescent (ECL) Western blotting detection system (Amersham) according to the manufacturers' instructions.

Dot blotting

Dot blotting was performed by transferring samples to nitrocellulose (Transblot, BioRad) with a 96-well Bio-Dot microfiltration apparatus (BioRad). The nitrocellulose was cut to size, soaked in PBS and placed on 3 MM paper (Whatman, Maidstone, UK), before being clamped into the apparatus. Samples containing 50 µg of purified canine IgG or whole, heat-inactivated (56 °C, 4 h) dog serum low in IgE and doubling dilutions thereof, were applied and drawn through the nitrocellulose by suction. Each well was washed twice with PBS. The membrane was then blocked with PBS-Tween (30 min, rt) and incubated overnight at 4 °C with rabbit anti-canine IgE (1/3000 in PBS-Tween). Antibody binding was detected as described for Western blotting.

RESULTS

Characterization of rabbit anti-canine IgE antisera

The specificity of the anti-canine IgE antisera was confirmed by both a reverse cutaneous anaphylaxis and a neutralizing PK test. Table 1 shows the ability of the whole anti-canine IgE serum to elicit reversed cutaneous anaphylaxis in 2 normal dogs. Included as controls were an anti-canine IgG antiserum and normal rabbit serum. The anti-IgE serum but not the control sera elicited a positive weal and flare response to a dose of 0.25–0.025 mg.

The rabbit anti-canine IgE serum was also tested, both before and after boosting with the human myeloma IgE, for its ability to produce a reduction in PK reactivity to flea antigen in a normal dog (Table 2). Serum obtained

Table 1. Ability of rabbit anti-canine IgE serum to elicit reversed cutaneous anaphylaxis

Amount (mg)	Normal rabbit serum	Rabbit anti-canine IgG	Rabbit anti-canine IgE
250	0/0	2+/2+	4+/4+
25	0/0	0/0	3+/3+
2.5	0/0	0/0	3+/2+
0.25	0/0	0/0	3+/2+
0.025	0/0	0/0	2+/0

prior to the myeloma boost (IgE-pre) reduced the PK titre from 1/2560 to 1/320. Post-boost serum (IgE-post), had an even more striking effect since, after use in absorbing Ruf serum, PK activity was not detectable at a dilution of 1/10.

To exclude residual reactivity of the absorbed IgE-antiserum to other immunoglobulin isotypes, a dot-blot immunoassay was performed using both purified dog IgG and a whole canine serum low in IgE which had been heat-inactivated, as antigen. No reactivity to either was observed to a dose of 50 µg per spot (data not shown).

Identification of IgE-binding components of the flea by immunoblotting

Sera from 30 dogs with suspected allergy to flea bites were examined for IgE binding to whole flea extract separated by SDS-PAGE and electroblotted onto nitrocellulose. Of the many resolved polypeptides in the MW range of 5–200 k, 16 (MW 14, 17, 25, 27, 28, 29, 31, 35, 37, 40, 43, 45, 58, 70, 100 and 150 k) bound canine IgE antibodies as

Table 2. PK neutralizing ability of rabbit anti-canine IgE Sera

Dilution	PK titre on absorption of Ruf serum with		
	Normal rabbit serum	Rabbit anti-canine IgE-pre	Rabbit anti-canine IgE-post
1/10	4+	2+	0
1/20	4+	2+	0
1/40	4+	2+	0
1/80	4+	1+	0
1/160	4+	1+	0
1/320	4+	1+	0
1/640	2+	0	0
1/1280	2+	0	0
1/2560	1+	0	0
1/5120	0	0	0

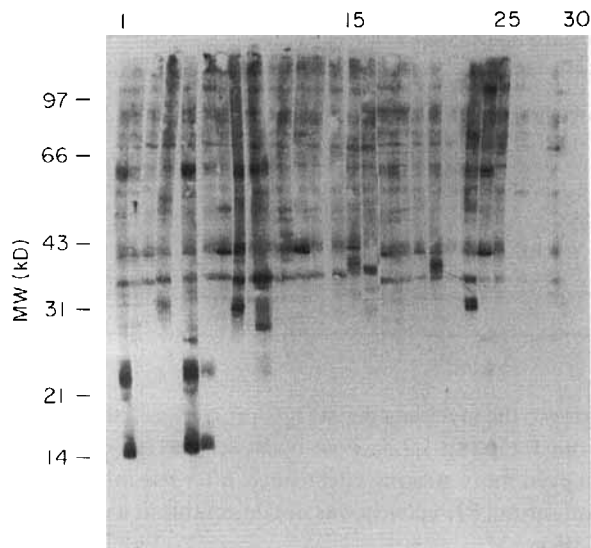


Figure 1. Identification by enhanced chemiluminescence of flea IgE-binding components transferred to nitrocellulose after SDS-PAGE. Strips 1–25 were incubated with sera from dogs with suspected flea allergy. Strips 26–30 were incubated with sera from known flea skin test-negative dogs.

measured by an enhanced chemiluminescent assay. One third (10) of the sera showed no IgE binding. One serum bound only 1 component and the maximum number of bands bound by a single serum was 9. The majority of the sera bound to between 2 and 5 components. An example of an IgE immunoblot showing the reactivity of 25 sera from suspected flea allergic dogs (lanes 1–25) and 5 sera from known flea skin-test negative dogs (lanes 26–30) is shown in Figure 1. Sera from dogs not allergic to flea bites did not react with any of the transferred polypeptides. In addition, no binding was observed when the anti-canine IgE was used alone (data not shown).

No 2 of the 20 positive sera showed the same pattern of IgE reactivity which demonstrated a high level of heterogeneity in the canine immune response to flea allergens. Moreover, the occasional strong binding of sera to certain components recognised at low frequency (e.g. sera 13 and 17 binding to the 150 K component) suggests that although some flea components represent unimportant allergens to a flea-allergic dog population as a whole, at an individual level they may be very important.

Interestingly, the binding of canine IgE to at least 1, and possibly 2, of the lower molecular weight components was almost exclusively associated with binding to other components on the same strip. For example, IgE binding of the 35 K band was always associated with reactivity of 31 and 29 K bands. This may possibly be due to

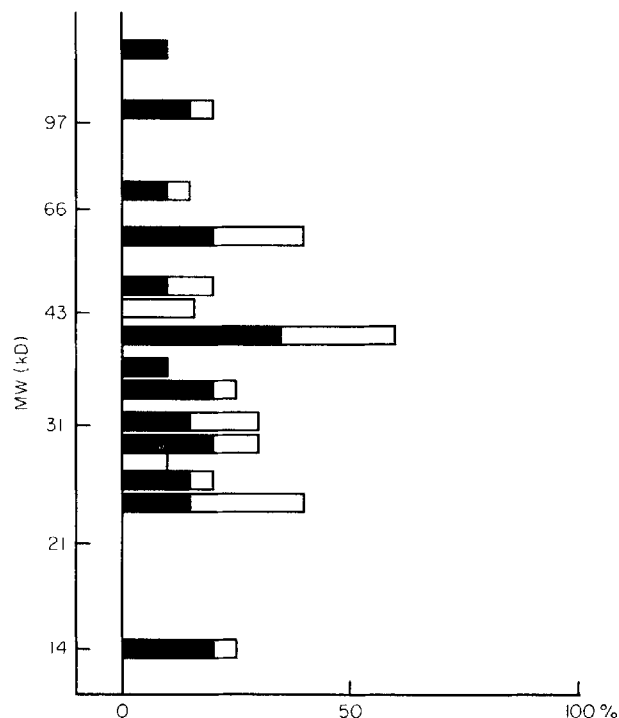


Figure 2. Histogram showing frequency of binding of IgE antibody by the 20 allergic dog sera to SDS-PAGE separated components of *C. felis* extract. A total of 15 IgE-binding components of whole flea extract were identified distributed in the MW range of 14 to 150 K. The percentage of sera bound weakly (□) to a component is illustrated along with the percentage of sera bound strongly (■).

degradation products of a single higher molecular weight component or, alternatively, may suggest that these bands represent separate components which possess shared allergenic determinants.

Frequency of IgE reactivity of flea components

The frequency of reactivity of the 15 IgE-binding components identified varied from 10 to 60% with no components being bound by all sera. The 40 K band was predominant and bound with 60% of the positive sera tested (Figure 2). Two further components of MW 25 and 58 K respectively, were also bound by at least 40% of the sera. Together, at least 1 of these 3 components was bound by 17 of the 20 sera (85%). Reactivity to the other components was less frequent and, in some cases, sporadic.

DISCUSSION

By IgE immunoblotting, we have identified allergenic polypeptides present in a whole extract of the cat flea,

Ctenophalides felis. Following separation by SDS-PAGE, at least 15 components reacted with IgE antibodies in the sera of dogs allergic to flea bites. Components of 25 K, 40 K and 58 K appeared to be recognized with the highest frequency, each reacting with at least 40% of the sera. These complex and markedly heterogeneous allergen recognition patterns are similar to IgE antibody responses observed in human subjects allergic to a variety of inhalant allergens (Tovey & Baldo 1987). Such heterogeneity in immune responsiveness is most likely to have a genetic basis. One important genetic influence on antibody repertoire has been shown to be genes linked to the major histocompatibility complex (MHC) (Berzofsky 1985). IgE responses, in particular, have been demonstrated to be heavily T-cell, and hence, MHC-dependent (Ishizaka 1984). Indeed, human studies have shown a relationship between the MHC and specific IgE responses (Marsh *et al.* 1982), and recent studies of the IgE antibody response to an allergen of the parasitic nematode, *Ascaris*, have revealed a striking MHC-restriction (Kennedy *et al.* 1991).

However, unlike complex allergen sources such as the house dust mite (Platts-Mills & Chapman 1987), whereby one or a few components are able to react with the majority (around 80–90%) of the allergic sera, none of the flea components was observed to react with a frequency of more than 60%. It would appear, therefore, that the allergenic activity of flea extracts is due to the presence of a number of antigenically distinct allergens and not due to any one dominant component. In fact, if the term 'major allergen' is applied, which was defined by Lowenstein (1978) as a component that binds IgE from at least 50% of the sera from an allergic population tested and which demonstrates strong binding in at least 50% of these cases, then this study has shown that only a single component of 40 K present in whole flea extracts is worthy of major allergen status.

Earlier studies performed using guinea pigs suggested that a low molecular weight hapten in flea saliva was responsible for the allergic reaction resulting from a flea bite (Benjamini *et al.* 1961). However, the presence of an immediate hypersensitivity reaction in this model suggested that larger IgE-binding antigens must also be present. Indeed, subsequent studies identified components of greater than 20 K in size, although these were not characterized in any detail (Halliwell 1984, Kwochka 1987). In the present study, we have demonstrated the presence of IgE specific for a number of flea polypeptides in the sera of dogs allergic to fleas but not in non-allergic controls. Although earlier reports indicated a significant involvement of a delayed or cell-mediated response (Benjamini *et al.* 1961, Gross & Halliwell 1985) the

present data suggest that immediate (IgE-mediated) hypersensitivity may also represent an important mechanism in the pathogenesis of canine flea allergy.

The identification of the allergenic components of the flea represents an important initial step in efforts to produce a standardized extract for both diagnosis and possible immunotherapy. In particular, the identification of the allergenically important components will ultimately allow the application of recombinant DNA methodology for the clonal production and detailed physico-chemical definition of these molecules.

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