

Putative salivary allergens of the cat flea, *Ctenocephalides felis felis*

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Abstract

The cat flea, *Ctenocephalides felis felis*, is the major initiator of flea bite hypersensitivity in dogs. Previous analyses of whole extracts of the flea and flea salivary secretions have failed to identify the allergens responsible. We dissected >2000 salivary glands from adult female fleas, extracted them into buffered saline containing protease inhibitors and fractionated the extract using gel permeation HPLC. Dogs were classified as hypersensitive to fleas (flea-feeding positive, FF+) or insensitive (flea-feeding negative, FF–) using a provocative test with live fleas. The allergenicity of the components of the salivary gland extract was tested by intradermal injection of samples of the column eluates. Dogs were also injected intradermally with a sample of whole salivary gland extract, and with histamine as a positive control. Negative control injections consisted of eluate from the column collected prior to fractions containing any protein. The skin of FF– dogs either did not respond or had a minimal response (a bleb ~2 mm larger than the injection blebs at the negative control injection sites) to all fractions and to the whole extract; histamine control injections produced positive responses (defined as wheals 5 mm greater than the blebs at the negative control injection sites) in all dogs. The skin of three of the nine FF+ dogs reacted positively to injection of a fraction containing protein/s with apparent MW 40k. Five other FF+ dogs reacted positively to the fractions containing proteins with apparent MW 12–8k. A single dog responded with very large, red wheals to injection of both the ~MW 40k and MW12–8k fractions. These findings suggest that proteins with apparent MW 40k and MW 12k–8k are important in flea bite hypersensitivity. This work also supports a previous finding that mice which had been exposed to flea bites had antibodies to proteins with ~MW 40k that were detected in salivary secretions of the flea. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *Ctenocephalides felis*; Cat flea; Insect allergens; Canine hypersensitivity; Insect saliva; FBH; FAD; Intradermal skin tests

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1. Introduction

Flea bite hypersensitivity (FBH) is a major cause of allergic dermatitis in dogs, yet relatively little has been reported on the allergens responsible. Early research using the oral secretions of the cat flea, *Ctenocephalides felis felis*, implicated a low molecular weight (MW) hapten as important in the immune responses of guinea pigs (Benjamini et al., 1960, 1963; Young et al., 1963); however, the immune responses of dogs to these secretions were not examined. Later studies assessed whole flea extracts using electrophoresis and immunoblotting with dog sera (Halliwell et al., 1987; Greene et al., 1993; Stolper and Opdebeeck, 1994; McKeon and Opdebeeck, 1994) but failed to identify specific flea allergens. Flea salivary secretions and extracts of whole salivary glands were used as antigens to study the antibodies raised in mice exposed to feeding fleas (Lee et al., 1997). The recognition by mice sera of protein bands with apparent MW of 56k, 54k, 42k and 40k suggested that these proteins are important antigens secreted by biting fleas. More recently it has been reported in conference proceedings and a patent application that some fractions of flea salivary secretions, separated by reverse phase high pressure liquid chromatography, induced hypersensitive skin reactions in six dogs exposed to fleas (Frank et al., 1996, 1997). Fractions containing predominant proteins with MW 42, 43 and 44k and MW 9 and 12k were suggested as possibly significant in flea allergy.

Here, we describe processes used to extract and fractionate flea salivary glands. We tested these salivary gland fractions for allergenicity by intradermal injection of dogs that were either hypersensitive to flea bites or insensitive. Those fractions that contained putative flea allergens of importance in FBH were identified.

2. Materials and methods

2.1. Preparation of salivary gland extracts

Fleas were reared using the system described by Lee et al. (1995). More than 2000 salivary glands were dissected from ca. 600 unfed adult female fleas (≤ 3 days post-emergence). Glands were placed into 100 μ l 0.1 M phosphate buffered saline (PBS) pH 7.2 containing protease inhibitors (Boehringer Mannheim Protease Inhibitors Set) in a microfuge tube, maintained on ice. Glands were disrupted by vortexing the tube for 15 s, centrifuging at $13\,600 \times g$, and sonicating (on ice) for 5 min (Unisonics Pty, Australia). This extraction procedure was repeated twice. The extract was stored at -70°C pending fractionation on a gel permeation column.

Whole extracts of smaller numbers of salivary glands (100 salivary glands/50 μ l representing $\sim 2 \mu\text{g}$ protein/50 μ l) were prepared for use in skin tests. These extracts were also stored at -70°C .

2.2. Gel permeation chromatography

A Superdex 200 HR 10/30 column was used to fractionate the proteins in the salivary gland extract. PBS was pumped through the column at a rate of 0.5 ml/min using a LC

1110 HPLC Pump (ICI Instruments). Proteins were detected by a linear 206 PHD detector and fractions were collected into a BioRad Model 2110 Fraction Collector. The column was equilibrated with PBS; MW standards (Sigma; range MW 12.4k–160k) were used to establish a standard curve. Prior to application to the column, the salivary gland extract was centrifuged at $13\,600 \times g$ for 10 min to sediment insoluble material. The supernatant was applied to the column by an automatic injector and fractions (60×1 ml) were collected over a 2 h period. Fractions (1 ml aliquots) were stored at -70°C until used for skin testing.

2.3. Intradermal tests with fractions and with non-fractionated salivary gland extract

Sixteen mixed breed dogs were obtained from a local pound and housed in the Small Companion Animal Clinic, the University of Queensland, for a minimum of 10 days prior to testing. For intradermal testing, dogs were premedicated with atropine sulphate (0.65 mg/ml, 0.5 mg/10 kg injected intravenously) before being lightly tranquillised with xylazine injected intramuscularly (20 mg/ml; 0.05–0.1 5ml/kg). A site was shaved on the lateral thorax and spots marked on the skin at regular intervals with a felt pen to define 26 injection sites, and a site for controlled flea feeding. All dogs were tested for hypersensitivity to fleas using a provocative live flea feeding test (Lee et al., 1995) and classified accordingly as flea-feeding positive (FF+; nine dogs) or flea-feeding negative (FF–; seven dogs). Samples (50 μl) of the following were injected intradermally using 27 gauge needles and 1 ml syringes: fractions collected from the column prior to the elution of any protein at sites 1–3 (negative controls); subsequent protein-containing fractions eluted from the gel permeation column at sites 4–24 (a single 50 μl injection of each fraction); histamine (1 : 100 000 dilution) (positive control) at site 25; and unfractionated whole salivary gland extract at site 26. Reactions were assessed 15–30 min after injection. The diameter of the wheals on both the long and short axes was measured with digital calipers (Mitutoyo) and an average value of the sum of the measurements was calculated. Wheals ≥ 5 mm larger than the blebs at the negative control injection sites were considered to be positive (Stolper and Opdebeeck, 1994). The bite site where 20 previously unfed adult fleas were exposed to the dogs' skin for 15 min was assessed at 30 min for swelling and reddening. Fleas were frozen after the test and, subsequently, squashed onto blotting paper to detect ingested blood to confirm that they had fed.

3. Results

The chromatogram of the fractionated extract of 2350 salivary glands revealed protein peaks at $\sim$ MW 56k, 40k, 12k and 8k and multiple peaks with MW < 8 k (Fig. 1(a)). The absorbance at 280 nm for the peak at $\sim$ MW 40k indicated a protein concentration of ~ 5 μg protein/ml. Typical skin responses of three FF+ dogs to the fractions tested are plotted below the chromatogram of the separated salivary proteins (Fig. 1(b)–(d)). The skin of dogs FF+#1 (Fig. 1(b)) and FF+#2 (Fig. 1(c); Fig. 2(a)) responded positively to the fractions that corresponded to the peak detected at apparent MW 40k (fractions 10–12). Another FF+ dog showed only a slight reddening and swelling at the site of injection

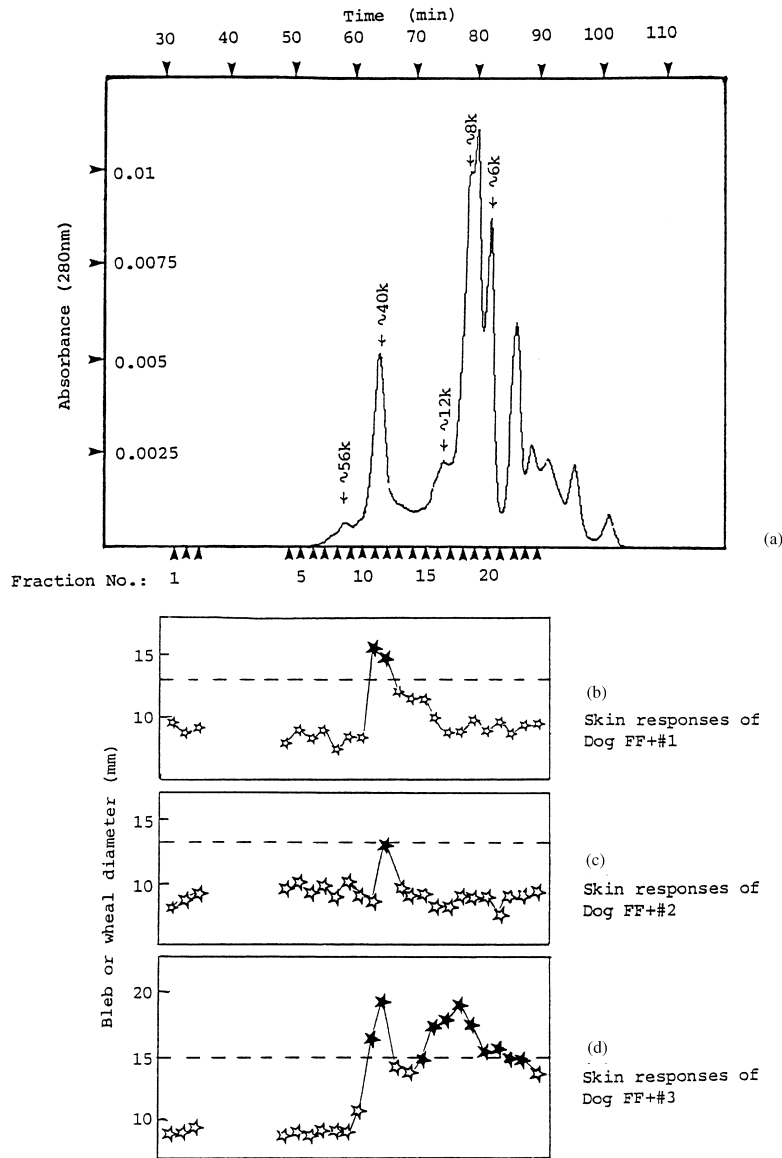


Fig. 1. Responses of the skin of three dogs identified as hypersensitive to flea bites (FF+) to intradermal injection of fractions of flea salivary gland extract separated by gel permeation HPLC. (a) Chromatogram of flea salivary gland extract fractionated by gel permeation HPLC on a Superdex 200 column. Fractions are designated by ▲▲. Fractions used were numbered 1–24 from left to right; 50 μ l portions of these fractions were used in IDT. (b)–(d) Skin responses of three FF+ dogs to intradermal injection of 24 of the fractions of salivary gland extract separated by gel permeation HPLC. Key: - - - positive cutoff (5 mm larger than the mean diameter of the blebs formed at the negative control sites (fractions 1, 2 and 3); ☆, blebs with diameter < positive cutoff that formed within 30 min of injection of fractions; ○, wheals with diameter $\geq$ positive cutoff that formed within 30 min of injection of fractions.

of fraction 12 (~MW 40k peak) and no other skin responses greater than those seen at the negative control injection sites. The skin of dog FF+#3 (Fig. 1(d); Fig. 2(b)) showed extreme sensitivity to the MW 40k peak, a lessening of the response to fractions 13–15

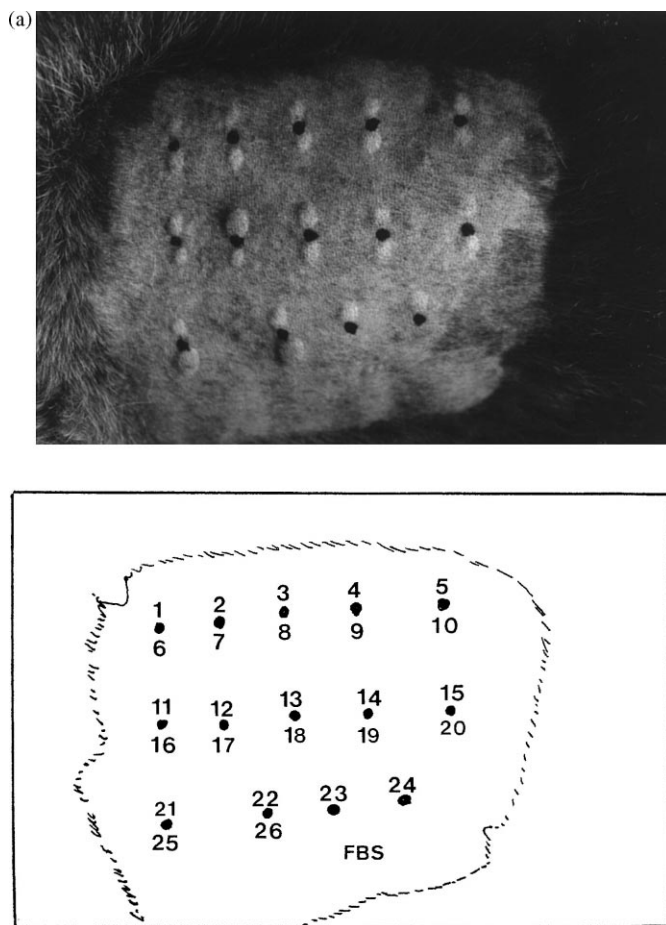


Fig. 2. Reactions of the skin of FF+ dogs to intradermal injection of fractions of flea salivary glands (see Fig. 1), histamine and whole extract of 100 glands. Injection sites were marked with a felt pen and 50 μ l injections administered above and below the marks. A gauze-covered tube containing live fleas was held on a shaved area of the dog's skin for 15 min. Sites were assessed for reactions at 15–30 min. (a) The skin of dog FF+#2 showed a slightly reddened, raised wheal in response to injection of fraction 12 which contained proteins with apparent MW ~40k. Injection of histamine, whole extract of 100 salivary glands and flea bites also caused a positive skin response. (b) The skin of dog FF+#3 showed extreme sensitivity to fraction 12 which contained proteins with apparent MW~40k and to fractions 16–19 containing proteins with MW 12–8k. Histamine, whole extract of 100 salivary glands and flea bites also caused markedly positive responses. Key to the layout of injection and flea bite sites in Fig. 2. For details of the fractions, refer to the chromatogram in Fig. 1. Sites 1–3: 50 μ l of fractions 1–3 injected as negative controls, Sites 4–24: 50 μ l of fractions 4–24 injected, Site 25: 50 μ l of positive control injected (histamine at 1 : 100 000), Site 26: 50 μ l of whole extract of 100 salivary glands, FBS Flea bite site.

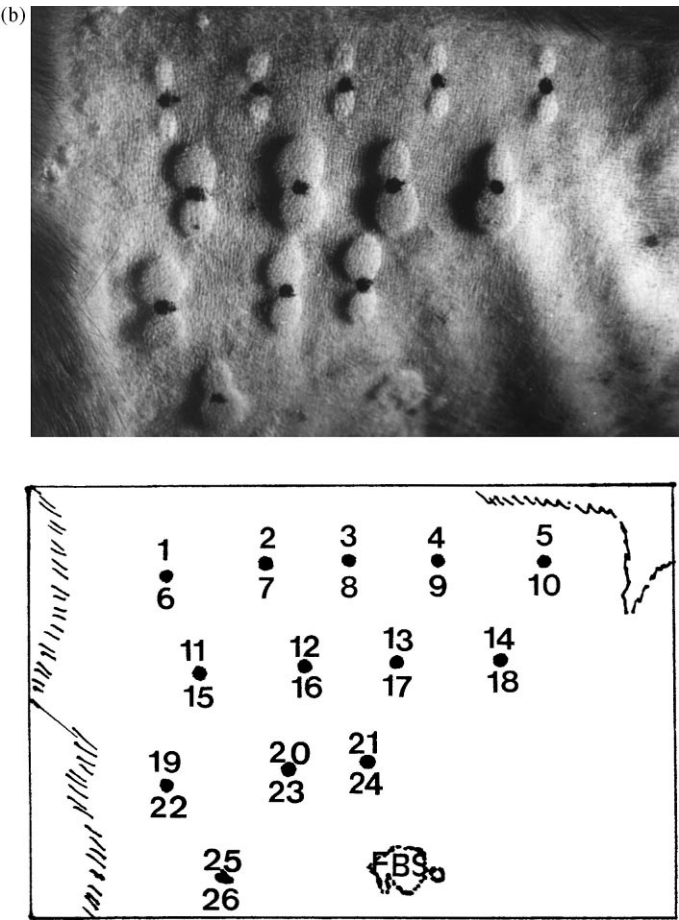


Fig. 2. (Continued)

(which had little protein present), a hypersensitive response to fractions 16–19 which contained proteins of ~MW 12–8k, then a diminished response to the remaining fractions tested. Five other FF+ dogs showed skin hypersensitivity only to fractions 14–17 which corresponded to the peak containing proteins with ~MW 12k. Six of the seven FF– dogs showed no hypersensitivity to any of the protein-containing fractions. One FF– dog which had scaly, greasy skin did not demonstrate hypersensitivity to feeding fleas but had skin which showed slight reddening and swelling towards injection of fractions 15–17 (~MW 12k). The reaction to the histamine control injection was positive in all cases. The skin of all FF+ dogs reacted positively to an injection of whole extract of 100 salivary glands; responses were $\geq$ positive responses to individual fractions. The skin of all FF– dogs either did not respond positively to the injection of whole extract of 100 salivary glands (blebs at injection sites were no larger than those at the negative control injection

sites) or showed some reddening and slight swelling (~ 2 mm larger than the blebs at the negative control injection sites) at the site of injection of the gland extract.

4. Discussion

Intradermal testing with salivary gland extracts, both whole and fractionated by gel permeation chromatography, revealed differences in the responses of dogs that were hypersensitive to flea bites and those that were insensitive. The chromatogram of separated salivary gland proteins revealed peaks containing small amounts of protein at $\sim$ MW 56k, 40k, 12k and ≤ 8 k. The peak at $\sim$ MW 40k had a protein concentration of ~ 5 μ g/ml (based on the absorbance reading at 280 nm), hence injection of 50 μ l of this fraction introduced only ~ 250 ng of protein intradermally. Nonetheless, this induced a hypersensitive skin response in four of the nine FF+ dogs tested; for three of these animals, reactivity was restricted to the $\sim$ MW 40k peak, suggesting that this fraction contained a protein or proteins of significance in FBH. A fourth FF+ dog, which was extremely sensitive to feeding fleas, showed strong reactivity to fractions encompassing this peak in addition to reactivity to fractions containing proteins with $\sim$ MW 12–8k. This indicates either that there are several flea allergens or that the MW 12–8k fraction contained breakdown products of the MW 40k fraction at sufficient concentration to trigger a response in an apparently highly sensitised dog. The former suggestion of a spectrum of allergens is supported by the finding that five other FF+ dogs which showed skin reactivity towards the fractions containing proteins with MW 12–8k showed no skin reactivity towards the $\sim$ MW 40k fraction. Proteins with $\sim$ MW 12–8k may therefore also be of significance in FBH.

In a patent application, Frank et al. (1997) reported the results of intradermal tests with a whole saliva extract obtained by eluting flea saliva products off a membrane which fleas had been able to probe for up to 72 h. They subjected two flea-naïve control dogs and six previously flea-naïve dogs, which they exposed to fleas in a controlled intermittent manner, to IDT with fractions of the membrane washings which had been separated by reverse phase HPLC. Based on positive results of intradermal tests for two of the six exposed dogs, they identified Fraction H, which contained predominant proteins of MW 9k and 12k, as possibly significant in flea allergy. In the study reported here which used nine dogs known to be hypersensitive to flea bites, the response of six out of nine FF+ dogs to the fractions of salivary gland extract containing proteins with $\sim$ MW 12k–8k support the suggestion that proteins with MW 12–9k may be significant in FBH. In the patent application, a fraction with predominant proteins of MW 42k, 43k and 44k (Fraction N) was also identified as possibly significant in flea allergy (Frank et al., 1997). All six dogs that had been exposed artificially to fleas gave positive responses (objectively measured and also subjectively graded as 3+ responses) to intradermal injection of Fraction N. This fraction may correspond in content to the fraction containing proteins with $\sim$ MW 40k identified in the study reported here. The MWs of the three predominant proteins in Fraction N (MW 42k, 43k and 44k) are also close to the MW of proteins previously identified as significant in the induction of antibodies to flea salivary gland proteins in mice exposed to feeding fleas (Lee et al., 1997).

Meaningful interpretation of results reported in the patent application (Frank et al., 1997) is limited, however, because it is unclear whether any of the six dogs described as sensitised to fleas suffered from FBH. To sensitise dogs, flea-naïve dogs were exposed once weekly to 25 contained-fleas that were able to feed on the dog for 15 min during the weekly exposure; the number of exposures was either 38, 22, 20, 17, 12 or once only. It was not stated whether any of these animals developed clinical FBH and their sensitivity to provocative feeding was not tested. In addition to positive responses by some or all of the exposed dogs to fractions containing predominant proteins with apparent MW 40k, 12k and 9k, numerous other samples containing proteins with MW between 6k and 55k also induced apparently hypersensitive responses in several of the dogs artificially exposed to intermittent flea feeding. Even the dog exposed only once to 25 contained-fleas for a 15 min period showed a 3+ skin reaction to injection of Fraction N and to a whole extract of salivary secretions when tested four months after the single exposure. This result implied that very little flea allergen applied in a single short exposure is needed to stimulate production of IgE antibodies; however, exposed dogs may not suffer FBH. Halliwell et al. (1987) found that the pattern of exposure to fleas influenced the probability that dogs would develop FBH. Those animals with continual exposure to fleas were less likely to develop hypersensitivity than those that received intermittent exposure. For the work reported in the patent application (Frank et al., 1997), the group of dogs showing positive responses to many components of flea salivary secretions were exposed to fleas intermittently and their immune responses may not reflect the responses seen in animals exposed naturally to fleas. In the work reported here, all the dogs (including negative control animals) had been exposed naturally to fleas and they were classified as hypersensitive to flea bites (FF+) or insensitive (FF–) by the response of their skin to a provocative challenge with live fleas. Hypersensitive responses seen in the FF+ dogs are more likely to correctly identify putative flea allergens relevant to FBH than responses seen in dogs exposed intermittently to short periods of flea feeding.

Injection of 50 µl of whole extract from 100 salivary glands produced a response in the skin of FF+ dogs at least as large as any of the positive skin responses to individual fractions. This may reflect a cumulative response to several allergens of different MW present in the whole extract, compared to the response to fewer allergens with particular MW encountered in a fractionated extract; alternatively the concentration of allergen/s in whole extract may have been greater than in the fraction containing that allergen. The response to whole extract is not significantly mediated by non-specific inflammatory mediators since a relatively small response was seen in the skin of FF– dogs.

5. Conclusion

The study presented here suggests that proteins with apparent ~MW 40k and 12–8k, present in fractionated extract of flea salivary glands and salivary secretions, provoke hypersensitive skin responses in dogs identified as being hypersensitive to flea bites. Some dogs reacted to only one fraction in an intradermal test while others reacted to several fractions. However, fractions which included proteins with either ~MW 40k or 12–8k consistently provoked a positive skin response in the FF+ dogs tested. Further

work is being done to separate and characterise single proteins which may be significant in flea bite hypersensitivity. Putative flea allergens isolated from flea salivary glands may provide superior test antigens for use in skin tests for FBH, or for use as desensitising agents; however, confirmation of their potential use as diagnostic or therapeutic agents will require production of recombinant proteins to provide sufficient quantities for comprehensive trials.

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