



RESEARCH NOTE

IgG AND IgE ANTIBODIES AGAINST ANTIGENS OF THE CAT FLEA, *CTENOCEPHALIDES FELIS FELIS*, IN SERA OF ALLERGIC AND NON-ALLERGIC DOGS

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Abstract—MCKEON S. E. & OPDEBEECK J. P. 1994. IgG and IgE antibodies against antigens of the cat flea, *Ctenocephalides felis felis*, in sera of allergic and non-allergic dogs. *International Journal for Parasitology* **24**: 259–263. Antigens from a soluble extract of cat fleas (FS) were separated using SDS–PAGE, and transferred to nitrocellulose paper. Sera from dogs shown by use of a provocative flea feeding test to be either allergic (23 dogs) or non-allergic (20 dogs) to flea bites, were used in Western blots to identify flea antigens that react with canine IgG or IgE. The sera were also tested in ELISA against FS to quantify levels of IgG and IgE antibodies. Antibodies present in the sera of both flea allergic and non-allergic dogs identified multiple antigens. There was a great diversity of responses within each group, and there was no pattern of reactivity that distinguished dogs with flea allergy from dogs not allergic to fleas. IgG and IgE antibodies were not significantly different between the two groups of dogs. These results demonstrated that there is little association between particular antibody responses and allergic reactivity of dogs to fleas.

INDEX KEY WORDS: Flea allergy dermatitis; *Ctenocephalides felis felis*; canine IgG; canine IgE; Western blot analyses; immunoblots.

THE cat flea, *Ctenocephalides felis felis*, is the most common cause of allergic dermatitis in the dog (Dryden & Blakemore, 1989). The clinical disease flea allergy dermatitis (FAD) results from hypersensitivity reactions to saliva of the feeding flea, exacerbated by self-trauma by the dog and by secondary bacterial infections. Earlier attempts to identify allergens were largely unsuccessful, although a low mol.wt hapten present in flea saliva and components with mol.wt greater than 18 k present in Greer flea extract were respectively implicated as causal agents by Young, Benjamini, Feingold & Noller (1963) and Halliwell, Preston & Nesbitt (1987). More recently during the preparation of this paper a study was published in which sera from 30 dogs, purported to suffer from FAD, were used to probe crude extracts of whole fleas in Western blot analysis (Greene, Carnegie, Shaw, Thompson & Penhale, 1993). IgE antibodies against a 40 k mol.wt component were detected in 12 sera from the dogs with FAD, and 17 of the 30 sera recognised at least one of three components at mol.wt 25, 40 and 58 k. The authors suggested that Type I hypersensitivity

against these antigens is an important mechanism in the pathogenesis of FAD. They also indicated that the IgE responses of individuals to the crude flea extract was more diverse than previously described (Greene *et al.*, 1993) although we note that Halliwell *et al.* (1987) had reported marked differences in the specificity of IgE responses on Western blots from 6 dogs allergic to fleas.

In previous work we demonstrated that positive reactions in an intradermal skin test (IDST) to the crude flea extracts available for use in IDST are not accurate evaluations of the flea-allergic status of an individual (Slacek & Opdebeek, in press; Stolper & Opdebeek, submitted). Accurate classification of animals as FAD positive or negative is particularly important for the purposes of research into the immunopathogenesis of the disease and we therefore have strongly advocated the use of a provocative test to establish the allergic status of dogs in experiments. A partially purified soluble extract of unfed adult fleas was found to have greater predictive value for FAD in an IDST than any of the other extracts tested (Stolper & Opdebeek, submitted) and we used this extract therefore to attempt to identify allergens of *C. felis felis* in Western blot analyses.

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Antisera were obtained from 23 dogs which reacted positively to feeding fleas (Type I hypersensitivity reactions following exposure to 20 fleas feeding for 15 min) (FF+) and from 20 dogs which did not react to feeding fleas (FF-) [see Slacek & Opdebeeck (in press); and Stolper & Opdebeeck (submitted) for details of the flea feeding assay and the experimental dogs, respectively]. The soluble flea extract was made from whole adult fleas using the method described by Slacek & Opdebeeck (in press). Briefly, about 7000 unfed fleas were disintegrated, homogenised and sonicated in 0.15 M-PBS, pH 7.2. The extract was centrifuged at 15,000 g, the pellet discarded and the supernatant centrifuged at 100,000 g. The supernatant thus obtained was designated FS. SDS-PAGE of FS was carried out using a discontinuous buffer system, with a 7.5% separating gel and a 4% stacking gel (Laemmli, 1970). Denatured FS antigens diluted to 1 mg ml⁻¹ were separated on the gel and stained using a combined Coomassie Blue-Silver stain procedure (De Moreno, Smith & Smith, 1985). Antigens for immunoblots were separated by SDS-PAGE and transferred by electrophoresis to nitrocellulose paper (ncp). The ncp was blocked with 5% low fat milk powder in PBS-Tween. Sera from FF+ dogs and from FF- dogs, diluted 1:30, were applied to the ncp. Bound IgG antibodies were detected using biotinylated rabbit anti-dog IgG (H&L chains specific) (Jackson ImmunoResearch Laboratories Inc. (JIRL)) followed by horse-radish peroxidase (HRPO) conjugated to streptavidin (JIRL) and then the substrate 4-chloro-1-naphthol. Bound IgE antibodies were detected using rabbit anti-dog IgE (H chain specific) (Halliwell & Longino, 1985) (kindly supplied by R.E.W. Halliwell) followed by a biotinylated-streptavidin-HRPO system and substrate as described above. IgG and IgE antibodies to FS were quantitated in ELISA using dog sera diluted 1:100. Rabbit anti-dog IgG (Organon-Tekniko Cappel) conjugated to HRPO was used as the marker system for measuring IgG antibodies to FS (15 µg ml⁻¹). The ELISA was amplified to increase its sensitivity to measure IgE antibodies. FS was applied to plates at 9 µg ml⁻¹ and amplification was achieved using rabbit anti-dog IgE (H chain specific) as the marker antibody, as described previously for immunoblots, followed by goat anti-whole rabbit serum (Cappel) and rabbit peroxidase-anti-peroxidase serum (Serotec). The substrate in both cases was 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS). Results (expressed as absorbance values) were read at 405 nm when a positive control serum reached a predetermined colour intensity. All reagents and antisera used in immunoblots and ELISA were first titrated in the relevant assay system to ensure that they

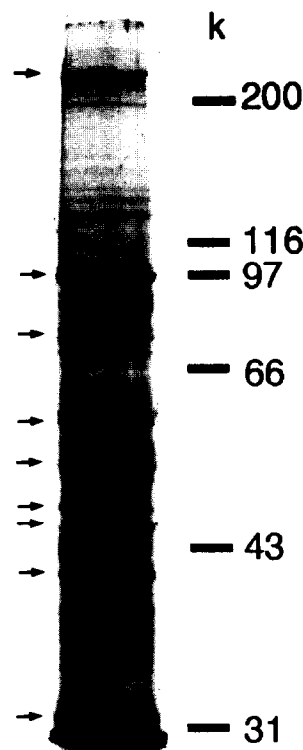


FIG. 1. Soluble extract of unfed adult fleas (FS) separated by SDS-PAGE (7.5% separating gel) and stained with combined Coomassie Blue-Silver protein stain.

were used at optimum concentrations.

There were nine prominent protein bands revealed by SDS-PAGE at mol.wt 224, 97, 72.4, 56.2, 50, 45.7, 44.6, 40.7, and 31.2 k (indicated by arrows in Fig. 1), and multiple other faint bands throughout the gel ranging in mol.wt from >200 k to <31 k (Fig. 1).

Multiple antigens were identified by both IgG (Fig. 2) and IgE (Fig. 3) antibodies from both FF+ and FF- dogs. There were no consistent differences in reactivity associated with either of the 2 groups of dogs for either of the 2 isotypes. The majority of dog sera tested reacted in a similar manner to antigens with mol.wt >66 k, irrespective of the allergic status of the dog. Bands at 224, 200 and 97 k were particularly strong. Below mol.wt 66 k there was a greater variation in reactivity of the dog sera than occurred at higher mol.wt, especially in the IgG blot (Fig. 2). Very few sera identified any antigens with mol.wt <37 k. Some antigens that were not prominent in the protein-stained gel were detected by antibodies in the dog sera, and most sera had an unique pattern of reactivity.

The distinctive features of the reactivity of both the IgG and IgE antibodies to FS were the diversity of response and the lack of a pattern of reactivity which distinguished FF+ from FF- dogs. Halliwell *et al.*

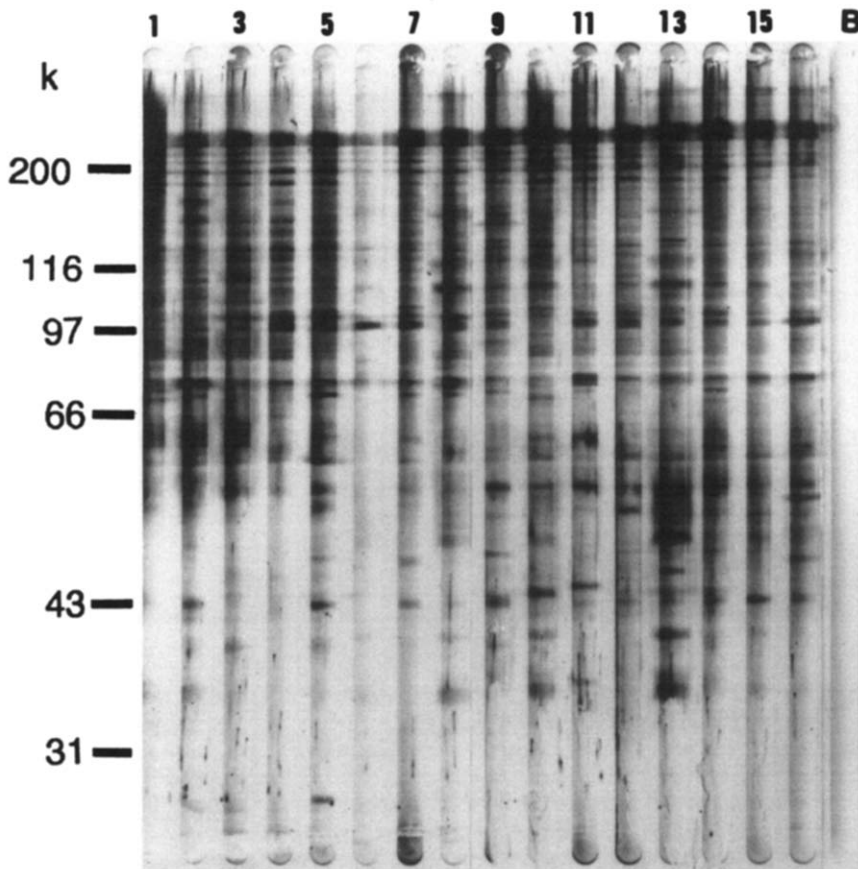


FIG. 2. Western blot showing reaction of IgG antibodies in different dog sera to soluble flea antigens separated by SDS-PAGE (7.5% separating gel). Dogs were either reactive to flea bites (FF + dogs) or non-reactive to flea bites (FF - dogs). Lanes 1-8: FF + dog sera; lanes 9-16: FF - dog sera; lane B: all reagents present except dog serum.

(1987) and Greene *et al.* (1993) have also reported a great diversity in the IgE antibody responses of individuals to putative flea allergens. Greene *et al.* (1993) suggested that a number of antigens may be responsible for allergy in dogs, and claimed that a molecule at 40 k was "worthy of major allergen status". This claim must be questioned if the definition of major allergens is accepted as "antigens which bind IgE from at least 50% of the sera of [allergic] patients tested and show strong binding to at least 50% of these" (Lowenstein, 1978). The 40 k molecule proposed by Greene *et al.* (1993) as a putative major allergen was recognised by only 12 of 30 dogs (40%) identified by the authors as having suspected allergy to flea bites. The authors appear to have calculated the frequency of binding of IgE antibody to the 40 k molecule (given as 60%) using only those 20 animals which reacted in their Western blot rather than the 30

dogs which they identified as having FAD based on one or more standard criteria. Unfortunately the reasons that 33% of flea allergic dogs were discarded from their calculations, and the possible relevance of the apparent lack of any anti-flea IgE antibodies in these sera are not discussed. It also appeared from the data presented that at least one of the 5 negative control dogs recognised the molecule with mol.wt 40 k. We and others have previously questioned the validity of IDST for diagnosis of allergy to fleas (Slacek & Opdebeeck, in press; van Winkle, 1981) and diagnosis of FAD on clinical presentation is conceded to be fraught with difficulties (Scott, 1981; Nesbitt, Kedan & Caciolo, 1984). We suggest that the 40 k molecule identified by Greene *et al.* (1993) might be more accurately evaluated for its potential as a diagnostic molecule if dogs for experimentation were first categorised for their allergic status to fleas using a

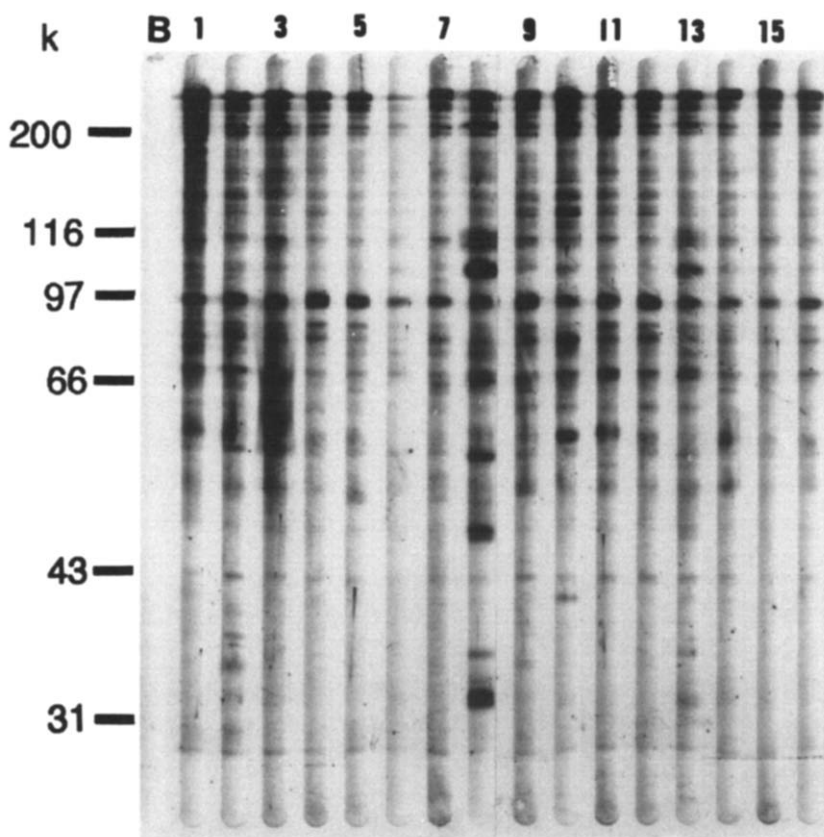


FIG. 3. Western blot showing reaction of IgE antibodies in different dog sera to soluble flea antigens separated by SDS-PAGE (7.5% separating gel). Dogs were either reactive to flea bites (FF+ dogs) or non-reactive to flea bites (FF- dogs). Dog sera are in identical order to those in Fig. 2. Lanes 1-8: FF+ dog sera; lanes 9-16: FF- dog sera; lane B: all reagents present except dog serum.

provocative test. In the study we describe here, dogs diagnosed with Type I hypersensitivity reactions in response to feeding fleas had neither IgG nor IgE antibodies that recognised any particular flea molecules more frequently or to a greater degree than the antibodies of dogs which were not responsive to feeding fleas. We could find no evidence of preferential reactivity by the FF+ group to a molecule with mol.wt 40 k, nor to bands at 25 or 58 k. Indeed we found that the number of FF+ dogs that reacted with these relatively low mol.wt antigens was much less than the number reacting with those antigens with mol.wt > 66 k. Both groups of dogs (FF+ and FF-) contained individuals that were strongly reactive (Figs. 2 and 3, lanes 8 and 13) and individuals that reacted poorly (Figs. 2 and 3, lanes 6 and 15); there were also individual dogs that reacted to bands at locations unique to that individual (Fig. 3, lane 8). We did find that sera from some dogs reacted with

components which were not prominent on the gel stained for protein; this, however, is not an unusual finding as allergens are effective at extremely low concentrations (Marsh, 1975) and this phenomenon had previously been commented on in relation to flea extracts by Halliwell *et al.* (1987). We could find no evidence of blocking IgG antibodies (Strannegard & Yurchuson, 1969; Tada & Okumura, 1971) in dogs that were not responsive to feeding fleas, although we are currently investigating this possibility further by selective absorption of isotypes from sera.

No significant difference was found in the ELISA for the level of IgG antibodies against FS between FF+ and FF- dogs using Student's *t*-test (Table 1). The difference in IgE anti-FS antibodies between FF+ and FF- dogs was significant at the 90% level of probability only (Table 1). The latter result may indicate a trend in the quantitative reactivity of allergic dogs to flea antigens and supports earlier work by

TABLE 1—IgG AND IgE ANTIBODIES AGAINST SOLUBLE FLEA ANTIGEN (FS) DETECTED BY ELISA; THE SERA OF DOGS ALLERGIC (FF+) AND NON-ALLERGIC (FF-) TO FLEA BITES WERE TESTED

Antibodies to FS	DOGS	
	FF+ (n=23)	FF- (n=20)
IgG ($\bar{x} \pm \text{s.d.}$)*	0.219 $\pm$ 0.029	0.210 $\pm$ 0.026
IgE ($\bar{x} \pm \text{s.d.}$)*	0.315 $\pm$ 0.164†	0.253 $\pm$ 0.076

*Absorbance units relative to positive control

†Significantly higher than FF- group at $p=0.1$; analyses carried out using Student's *t*-test.

Halliwell and Longino (1985). They found significantly higher levels of both IgE and IgG anti-flea antibodies in the sera of 47 flea allergic dogs compared to sera from 40 healthy pound dogs, using RIA to quantitate the antibodies. We are currently testing further for a possible relationship between the levels of antibodies and allergic status using groups of FF+ and FF- dogs, accessed through the Veterinary Clinic of The University of Queensland.

In view of the results obtained by Greene *et al.* (1993) and ourselves both in this and earlier studies it must be concluded that immunoblots and ELISA have revealed only tenuous associations between particular antibody responses and reactivity to fleas. More work is necessary to identify antigens with putative value in the diagnosis and therapy of flea allergy dermatitis.

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