

The significance of reactions to purified fractions of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* in canine atopic dermatitis

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Accepted 13 December 1995

Abstract

The significance of reactions to crude extracts and purified fractions of the house dust mites *Dermatophagoides pteronyssinus* (Der p I and Der p II) and *Dermatophagoides farinae* (Der f I and Der f II) was evaluated in dogs with clinical manifestations of atopic dermatitis (AD). In 13 healthy control dogs and eight dogs with AD, immediate skin test reactivity was determined to serial dilutions of Der p I, Der p II, Der f I and Der f II. In addition, allergen-specific IgGd antibodies were determined by means of an enzyme-linked immunosorbent assay (ELISA) and Western blots. The results suggest that, in contrast to what occurs in humans and despite immediate skin test reactivity in some dogs, Der p I, Der p II, Der f I and Der f II are unlikely to be major allergens in dogs with AD. However, only serum of atopic dogs consistently binds a 90 kDa polypeptide of *D. farinae*, as shown by Western blot analysis.

Keywords: *Dermatophagoides* spp.; Atopic dermatitis; Immunoblotting; Skin test; Dog

1. Introduction

House dust mites comprise two species that release allergens into the domestic environment and are major causes of allergic disease in people (Price et al., 1990; Peat

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et al., 1993; Ollier and Davies, 1994). Three groups of allergens, Group Der I, Group Der II, and Group Der III, have been isolated from each of the two species of *Dermatophagoides* mites: *pteronyssinus* and *farinae* (Tovey and Baldo, 1987; Chapman et al., 1987; Heymann et al., 1989; Stewart et al., 1989; Thomas et al., 1992). Within each group, allergens appear to be structural homologues, whereas allergens from the different groups seem antigenically unrelated. In mite-allergic individuals, immediate hypersensitivity has been demonstrated to both Group I and Group II allergens and these allergens are generally accepted as being major allergens (Van der Zee et al., 1988; Ollier and Davies, 1994). In contrast, it is questionable whether Der p III and Der f III should also be considered as major allergens (Tovey and Baldo, 1987; Thomas et al., 1992).

Also in dogs, house dust mites (*Dermatophagoides* spp.) play an important role in the pathogenesis of atopic dermatitis (AD) (Willemse, 1991). Immediate skin test reactivity to crude extracts of *D. pteronyssinus* and *D. farinae* has often been observed in dogs with clinical manifestations of AD. Geographic differences in the distribution of house dust mites are well known, but in dogs, skin test reactions are much more common to *D. farinae* extracts (18–80%) than to *D. pteronyssinus* (2–22%) (Willemse, 1987; Carlotti and Costargent, 1994; Koch and Peters, 1994).

In canine AD two types of immunoglobulin (Ig) are involved in the immunopathogenesis: IgE (Patterson et al., 1963; Halliwell and Kunkle, 1978) and IgGd (Willemse et al., 1985a). In addition to antigenic similarity between canine and human IgE (Patterson et al., 1969; Halliwell et al., 1972), and evidence for skin sensitizing properties of both a canine IgE-like antibody (Rockey and Schwartzman, 1967) and IgGd (Willemse et al., 1985a) by means of PCA and PK-tests, IgE receptors have been reported on mast cells of atopic dogs (Halliwell, 1973). Hence, immediate skin test reactivity is still the most appropriate 'golden standard' for detection of allergy in atopic animals. Depending on the allergens involved, the agreement between in vivo and in vitro allergy tests (serological determination of allergen-specific IgE or IgGd antibodies) ranges between 20 and 80% for both Igs (Halliwell and Kunkle, 1978; Kleinbeck et al., 1989; Willemse et al., 1985b).

Because only crude antigen extracts have been used for skin testing in dogs, it is unknown whether similar specific components of *Dermatophagoides* spp. contribute to immediate skin test reactivity, as they do in man. Therefore, the aim of this study was to investigate skin test reactivity to Der p I and II, and Der f I and II extracts, and to compare skin test reactivity with results of immunoblotting.

2. Materials and methods

2.1. Dogs

Twenty-one dogs were included in the study. Thirteen were clinically healthy dogs, including nine beagles, three mongrels and one boxer, 2–8 years of age (median 5.9 years). Eight of the healthy dogs were females. Eight other dogs were patients referred to the Department of Clinical Sciences of Companion Animals of Utrecht University.

Their ages ranged between 1.5 and 8 years (median 3.8 years), and five were females. These eight dogs fulfilled the clinical criteria for atopic disease (Willemse, 1987). At the start of the study all atopic dogs were free of ectoparasites and adverse food reactions were excluded by means of a lamb and rice diet for at least 6 weeks. All medications were withdrawn for at least 6 weeks prior to the first examination.

2.2. Sera

Blood was obtained by jugular venepuncture from all dogs, allowed to clot for 1 h, and centrifuged (2000 rev min⁻¹, 10 min). Sera were stored at -70°C until used.

2.3. Antigens

Crude extracts of *D. pteronyssinus* and *D. farinae* were obtained as lyophilised products (ARTU Biologicals, Lelystad, Netherlands) and stored at 4°C until required. Antigen solutions were prepared for use in immunoassays by diluting the lyophilised antigens in phosphate-buffered saline, pH 7.4 (PBS), these thereafter being dispensed in 1 ml aliquots and frozen at -70°C. The standard antigen stock solution for further use in the immunoassays had a concentration of 10 mg ml⁻¹ saline.

Antigens for skin testing were supplied with 0.1% phenol (ARTU Biologicals). Highly purified fractions of *D. pteronyssinus* (Der p I and Der p II) and *D. farinae* (Der f I and Der f II) in saline containing dog serum albumin (0.3 mg ml⁻¹), were kindly provided by Prof. dr R.C. Aalberse (Central Laboratory of Blood Transfusion of the Dutch Red Cross, Amsterdam, Netherlands).

2.4. Peroxidase-conjugated anti-canine IgGd

A previously described rabbit IgG-anti-canine IgGd (heavy chain specific) was used (Willemse et al., 1985b). Prior to using the antiserum to detect antibodies in the assay, it was conjugated with peroxidase (Boehringer, Mannheim, Germany) according to the manufacturer's instructions.

2.5. Enzyme-linked immunosorbent assay (ELISA)

For detection of IgGd and antibodies to both crude and purified mite-derived antigens, an indirect ELISA was used. The antigens (at 5 µg protein per well) in 0.1 M sodium carbonate buffer, pH 9.6, were coated on 96-well microtitre plates by incubation overnight at 4°C or for 2 h at 37°C. Following incubation the remaining active protein binding sites were blocked by incubation with 5% (w/v) skimmed milk in PBS. Serum samples from atopic dogs and controls were diluted serially (1:20 to 1:2560) to determine titres. Peroxidase-conjugated rabbit anti-canine IgGd was diluted 1:600. All dilutions were made in 5% skimmed milk in PBS and all incubations were performed at 37°C for 1 h. Between incubations, the wells were washed (four times) with tap water. The substrate used for colour development consisted of hydrogen peroxide (H₂O₂) and 2-2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), and plates were read at 405 nm

using a microtitre plate reader (Titertek Multiscan, Flow Laboratories, Ayshire, UK). An OD-405 nm reading higher than the mean conjugate background from eight antigen-containing wells (no serum added) ± 3 SD (≤ 0.150) was considered a positive ELISA test. Values are presented as titres, these being the reciprocals of the highest serum dilutions giving positive ELISA results.

2.6. SDS-polyacrylamide gel electrophoresis (PAGE)

Antigens and molecular weight markers (Rainbow Markers, Amersham, UK) were separated under reducing conditions in a 12% SDS-polyacrylamide running gel (one sample per gel). Samples were diluted in Laemmli sample buffer (LSB, $3 \times$ concentrated), stored for 5 min at 95°C and then loaded onto a 4% SDS-polyacrylamide stacking gel. Gels were run at constant voltage (minigel, 120 V for 1.5 h) and subsequently stained with Coomassie blue, or SDS-PAGE-separated polypeptides were electroblotted onto nitrocellulose.

2.7. Immunoblotting procedure

Antigen preparations were separated by SDS-PAGE as described above and the gel was washed in blotting buffer. Electrophoretic transfer to nitrocellulose was performed at constant current ($0.8 \times$ gel area in mA) for 1 h. Following electrotransfer of the SDS-PAGE-separated polypeptides, the remaining active protein binding sites were blocked with 5% skimmed milk in PBS. The membrane was cut into strips (15 strips per minigel) and each strip was washed (four times) with PBS. This washing procedure was repeated after all subsequent incubations. The membrane strips were incubated with the relevant dog sera (diluted 1:25) followed by a 1-h incubation with peroxidase-conjugated rabbit anti-canine IgGd diluted 1:600. All dilutions were made in 5% skimmed milk in PBS, and all incubations were performed at room temperature with gentle shaking. Specific antibody binding was visualised with the following substrate solution, i.e. H_2O_2 and 4-chloro-1-naphthol. The reaction was stopped by washing in distilled water.

2.8. Intradermal tests

Skin tests were performed as previously described (Willemse and Van den Brom, 1982). Briefly, 0.05 ml of each antigen was injected intradermally on the thoracic wall, after gently shaving off the hair. A 0.01% histamine solution served as positive control solution. Saline, a 0.1% phenol solution (the preservative in the antigen solution), and dog serum albumin were used as negative controls. Crude mite extracts were injected at a standard concentration of $10 \mu\text{g ml}^{-1}$. The purified fractions were injected in 10-fold dilutions: Der p I at 18.25, 1.85 and $0.18 \mu\text{g ml}^{-1}$; Der p II at 1.37, 0.14 and $0.01 \mu\text{g ml}^{-1}$; Der f I at 42, 4.2 and $0.42 \mu\text{g ml}^{-1}$; Der f II at 1.2, 0.12 and $0.01 \mu\text{g ml}^{-1}$. Skin reactions were read after 15–20 min. Wheals (measured in mm) less than half of the diameter of the positive control were considered negative (0), wheals between 50 and

75% were considered positive (1 +), and wheals more than 75% of that of the histamine solution were considered strongly positive (2 +).

3. Results

3.1. Intradermal skin tests

3.1.1. Control dogs (Tables 1 and 2)

None of the control dogs ($n = 13$) had positive reactions to any of the negative control solutions. In all cases, strong positive reactions were observed to the 0.01% histamine solution. None of the control dogs had reactions to the crude mite extract of *D. farinae* or *D. pteronyssinus*, nor to the Der p and Der f fractions, the concentrations of which were therefore suitable for testing in atopic dogs.

Table 1

Results of intradermal (ID) tests, allergen-specific IgGd ELISA and Western blots with *Dermatophagoides pteronyssinus* (Der p) extracts in healthy dogs (nos. 1–13) and atopic dogs (nos. 20–27)

N	ID tests					ELISA			Western blots		
	Crude ^a	I ^b	I ^c	II ^d	II ^e	Crude ^f	I ^g	II ^h	Crude ⁱ	I ^j	II ^k
1	–	–	–	–	–	< 1:20	< 1:20	< 1:20	–	–	–
2	–	–	–	–	–	< 1:20	< 1:20	< 1:20	–	–	–
3	–	–	–	–	–	< 1:20	< 1:20	< 1:20	65	–	–
4	–	–	–	–	–	< 1:20	< 1:20	< 1:20	–	55–60	68
5	–	–	–	–	–	1:160	< 1:20	< 1:20	–	–	–
6	–	–	–	–	–	< 1:20	< 1:20	< 1:20	65	–	–
7	–	–	–	–	–	1:40	1:40	< 1:20	65	–	–
8	–	–	–	–	–	< 1:20	< 1:20	< 1:20	–	–	–
9	–	–	–	–	–	< 1:20	< 1:20	< 1:20	–	–	–
10	–	–	–	–	–	1:160	< 1:20	< 1:20	65	–	–
11	–	–	–	–	–	1:80	< 1:20	< 1:20 >	70	–	–
12	–	–	–	–	–	< 1:20	< 1:20	< 1:20	–	55–60	–
13	–	–	–	–	–	1:160	< 1:20	1:40	–	55–60	–
20	–	–	–	++	0.01	1:640	< 1:20	< 1:20	65	–	–
24	++	–	–	–	–	1:2560	1:40	< 1:20	–	–	–
27	–	++	18.2	–	–	< 1:20	< 1:20	< 1:20	–	–	–

^a ID test results with crude extract.

^b ID test results with Der p I fraction.

^c Lowest concentration ($\mu\text{g ml}^{-1}$) of Der p I fraction eliciting a skin reaction.

^d ID test results with Der p II fraction.

^e Lowest concentration ($\mu\text{g ml}^{-1}$) of Der p II eliciting a skin reaction.

^f ELISA titre with crude mite extract.

^g ELISA titre with Der p I fraction.

^h ELISA titre with Der p II fraction.

ⁱ Reactivity bands with Western blots performed with the crude mite extract (in kDa).

^j Reactivity bands with Western blots performed with the Der p I fraction (in kDa).

^k Reactivity bands with Western blots performed with the Der p II fraction (in kDa).

The test results of the five remaining atopic dogs were all negative and therefore not separately included.

3.1.2. Atopic dogs

None of the eight atopic dogs had positive reactions to any of the negative control solutions, whereas reactions to the histamine solution were consistently positive. The various reactions to the crude and purified extracts of *D. pteronyssinus* and *D. farinae* are summarised in Tables 1 and 2.

3.2. ELISA

3.2.1. Control dogs

Allergen-specific IgGd antibody titres to crude extracts of *D. pteronyssinus* ranged from less than 1:20 to 1:160 (Table 1) and to *D. farinae* from less than 1:20 to 1:320

Table 2

Results of intradermal (ID) tests, allergen-specific IgGd ELISA and Western blots with *Dermatophagoides farinae* (Der f) extracts in healthy dogs (nos. 1–13) and atopic dogs (nos. 15–30)

N	ID tests					ELISA			Western blots		
	Crude ^a	I ^b	I ^c	II ^d	II ^e	Crude ^f	I ^g	II ^h	Crude ⁱ	I ^j	II ^k
1	–	–	–	–	–	1:80	< 1:20	< 1:20	68	–	–
2	–	–	–	–	–	< 1:20	< 1:20	< 1:20	–	–	–
3	–	–	–	–	–	1:80	< 1:20	< 1:20	–	–	–
4	–	–	–	–	–	< 1:20	< 1:20	< 1:20	68	68	–
5	–	–	–	–	–	< 1:20	< 1:20	< 1:20	–	–	–
6	–	–	–	–	–	1:160	< 1:20	< 1:20	68	–	–
7	–	–	–	–	–	1:160	< 1:20	< 1:20	68	–	–
8	–	–	–	–	–	< 1:20	< 1:20	< 1:20	–	–	–
9	–	–	–	–	–	1:160	< 1:20	1:40	68	–	–
10	–	–	–	–	–	1:320	1:40	1:40	68	–	–
11	–	–	–	–	–	1:160	< 1:20	1:40	68	–	–
12	–	–	–	–	–	1:80	< 1:20	< 1:20	–	68	–
13	–	–	–	–	–	1:160	1:40	< 1:20	68	–	–
15	++	–	–	–	–	1:2560	1:80	1:160	68, 90	–	–
16	–	++	42	–	–	1:2560	< 1:20	1:40	68, 90	–	–
18	–	++	42	–	–	< 1:20	< 1:20	< 1:20	–	–	–
19	+++	+++	42	–	–	1:640	< 1:20	1:40	68, 90	–	–
20	–	–	–	++	1.2	1:2560	1:160	1:40	68, 90	–	–
24	++	–	–	–	–	1:160	< 1:20	1:40	90	–	–
27	+++	++	4.2	++	1.2	< 1:20	< 1:20	< 1:20	–	–	–
30	++	–	–	–	–	1:1280	< 1:20	< 1:20	68, 90	–	–

^a ID test results with crude extract.

^b ID test results with Der f I fraction.

^c Lowest concentration ($\mu\text{g ml}^{-1}$) of Der f I fraction eliciting a skin reaction.

^d ID test results with Der f II fraction.

^e Lowest concentration ($\mu\text{g ml}^{-1}$) of Der f II eliciting a skin reaction.

^f ELISA titre with crude mite extract.

^g ELISA titre with Der f I fraction.

^h ELISA titre with Der f II fraction.

ⁱ Reactivity bands with Western blots performed with the crude mite extract (in kDa).

^j Reactivity bands with Western blots performed with the Der f I fraction (in kDa).

^k Reactivity bands with Western blots performed with the Der f II fraction (in kDa).

(Table 2). In none of the control dogs were allergen-specific IgGd antibodies found to Der p I, Der p II, Der f I, or Der f II (titres less than 1:20 to 1:40). Hence titres of 1:640 or more to crude mite extracts and of 1:80 or more to mite fractions were considered to be relevant in atopic dogs.

3.2.2. Atopic dogs

Of the three atopic dogs with positive skin test for *D. pteronyssinus*, two had elevated titres of 1:640 and 1:2560. None of the dogs had elevated titres to the purified fractions of Der p I or Der p II (Table 1). Five dogs had elevated allergen-specific IgGd titres (from 1:640 to 1:2560) using the *D. farinae* crude extract. Elevated titres to Der f fractions were observed in two dogs with anti-IgGd titres to Der f I (1:80 and 1:160) and Der f II (1:160) (Table 2).

3.3. SDS-PAGE Western blots

3.3.1. *D. pteronyssinus* (Table 1)

Four control dogs and one atopic dog reacted to a 65 kDa polypeptide. Serum of dog no. 11 (control) had a smear of reactions to polypeptides ranging from 70 to 97 kDa, with slight formation of bands. Three control dogs (nos. 4, 12 and 13) but none of the

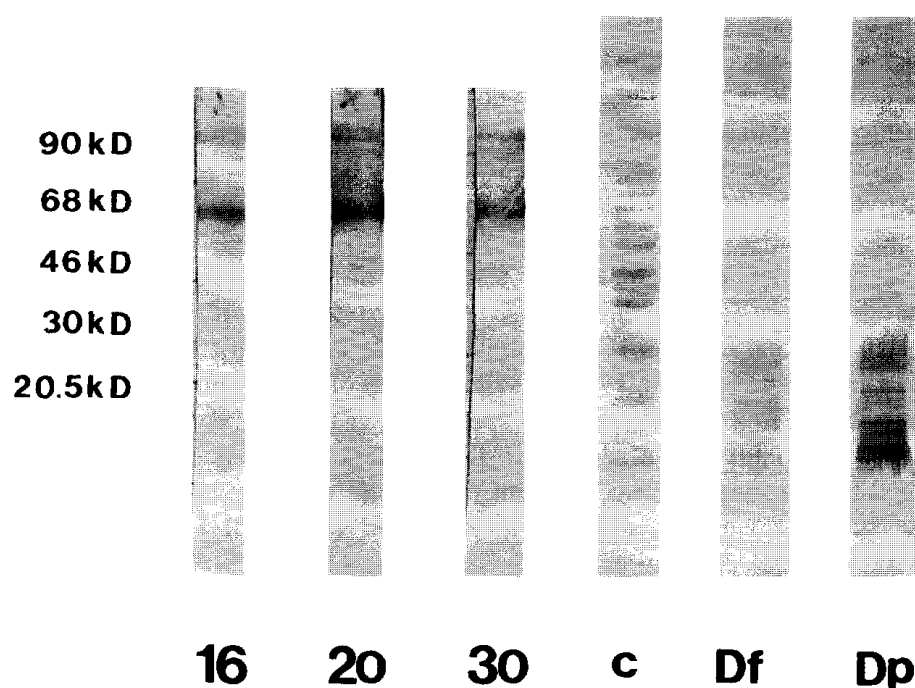


Fig. 1. Reactivity bands in Western blots performed with the crude mite extract of *D. farinae* in atopic dogs (nos. 16, 20 and 30) and SDS-PAGE control strips (c, control MW markers; Df, crude extract of *D. farinae*; Dp, crude extract of *D. pteronyssinus*).

atopic dogs had positive reactions to the purified fraction Der p I (55–60 kDa). One control dog (no. 4) had a light 68 kDa reaction band to the Der p II fraction.

3.3.2. *D. farinae* (Table 2)

Eight of the control and five of the atopic dogs had positive reactions (68 kDa band) in Western blots with the crude extract of *D. farinae*. Reactions to a 90 kDa band (Fig. 1) were seen in sera of six of the eight atopic dogs but in none of the control dogs. Two control dogs but none of the atopic dogs had a 68 kDa reaction band in Western blots with the purified fraction Der f I. No reactions were found in any animal in blots performed with Der f II.

4. Discussion

These results indicate that atopic dogs apparently do not recognise Der p I (25 kDa), Der p II (14 kDa), Der f I (25 kDa), or Der f II (14 kDa). This conclusion is based on two observations. First, in Western blots performed with crude extracts of the mites there were no visible bands, in either the 14 kDa or the 25 kDa region. Furthermore, no positive reactions were found by Western blot analysis using the Der p and Der f fractions and sera from atopic dogs. Secondly, IgGd specific for the latter fractions was not routinely found by ELISA in serum of atopic dogs, although it is noteworthy that two atopic dogs had low titres to Der f I and/or Der f II. These negative reactions were not due to the absence of mite-specific IgGd antibodies, as demonstrated by the presence of allergen-specific IgGd antibodies to crude mite extracts in these dogs. The results do not indicate that Der I or II antigens are not important in dogs, because only part of the possible allergic response, that is, the IgGd response and not the IgE responses to these antigens, was evaluated. For example, a discrepancy between positive skin test reactions and negative in vitro (IgGd) test results, may be explained by this. Positive in vitro results and a negative skin test may be attributed to a difference in sensitivity of the tests. The results of serologic allergy testing in dogs with atopic dermatitis (either determining allergen-specific IgE or IgG) correlate poorly with those of intradermal testing (Halliwell and Kunkle, 1978; Willemse et al., 1985b; Griffin, 1989; Kleinbeck et al., 1989; Codner and Lessard, 1993). The sensitivity of intradermal tests is 70–90% and of in vitro allergy tests 100%, but the specificity is respectively more than 90% and 0–50% (Scott et al., 1995). In addition, it is important to realise that serologic tests and intradermal tests screen for two different things: the presence of reagenic antibody in serum versus the detection of such antibody on mast cell surfaces. Hence it is questionable whether correlations may be expected. Although the intradermal injection of anti-canine IgGd in normal dogs results in wheal formation (Roosje et al., unpublished data, 1994), thereby supporting the role of IgGd as skin sensitizing antibody, the discrepancy between the skin test and the in vitro test may also indicate that in atopic dogs two types of homocytotropic antibodies can play a dominating role, e.g. in the dogs in this study mainly IgE.

When tested against the crude extracts of mites by Western blotting, atopic sera

recognised several other polypeptides of heavier molecular weight and different from Der p I, Der p II, Der f I, and Der f II fractions. It is interesting to note that the 90 kDa band observed on *D. farinae* blots was recognised only by atopic sera. It is therefore tempting to assume that this molecule might well be a specific allergen.

The results of this study confirm earlier observations (Esch, 1991; Bard and Esch, 1993) indicating that, in atopic dogs, Der p I, Der p II, Der f I, and Der f II are not recognised as major allergens by canine IgE antibodies. Using anti-canine IgE antibodies, the latter investigators found in *D. farinae* reactive animals a precipitin band at > 80 kDa by Western blot, and reported that only one subset of the canine population manifested such a reaction. Although the present study has used anti-IgGd antibodies, it is interesting that a 90 kDa band was also observed only when sera of atopic dogs were tested against *D. farinae* crude extract. One should bear in mind, however, that the anti-IgGd serum used was not absorbed with IgE (because such antibody is not commercially available) and hence the anti-IgGd serum described here may also recognise canine IgE. Hence the immunoblotting results could theoretically be explained by the concurrent presence of IgE, but this is improbable because previous immunoblotting studies using the present anti-IgGd antiserum and supposedly IgE-containing serum consistently failed to produce a 80 kDa band (IgE heavy chain).

In this study *D. pteronyssinus* reactions were found to be relatively unimportant in dogs with atopic dermatitis (AD), which is in accordance with existing data (Willemse, 1987; Carlotti and Costargent, 1994; Koch and Peters, 1994). In only two of the eight dogs with AD was there skin test reactivity to Der p I or Der p II and in only one dog was there reactivity to the crude extract. This may indicate that the amount of Der p I and Der p II in the routinely tested crude extract is too low to elicit skin test reactivity or that Der p I and Der p II are non-relevant fractions in dogs, as indicated by the immunoblotting results.

The observations concerning skin test reactivity to *D. farinae* crude extracts are in accordance with existing knowledge (Willemse, 1987; Carlotti and Costargent, 1994; Koch and Peters, 1994). In two dogs with AD, skin test reactivity was seen to Der f II and in four dogs to Der f I. However, immunoblotting did not reveal reactivity at relevant levels.

The reason for the discrepancy between skin test experiments using purified fractions and those using crude extracts of mites is not understood. A possible explanation is that the concentration of purified fractions in the skin tests was not sufficient to elicit a positive reaction even in highly allergic animals. However, it should be emphasised that the skin tests were performed with purified allergen concentrations 10–40 000 times higher than those used in human skin testing. Hence, it is most unlikely that the lack of reactivity was due to too low skin test threshold concentrations.

The observations that in some dogs skin test reactivity occurred to crude mite extracts and not to the respective fractions cannot be explained by a higher Der p or Der f fraction concentration in the crude extract. In the latter case, Western blots should have identified relevant polypeptides. The limited reactivity to Der p and Der f fractions in dogs with AD and the discrepancy between reactivity to crude mite extracts and mite fractions may indicate that Der p I, Der p II, Der f I, and Derf II fractions are of minor importance for dogs with AD and that other components may be responsible for eliciting

an allergic reaction. This was also indicated by the results of the IgGd-ELISA and immunoblotting, in which atopic dog-specific 90 kDa bands were observed.

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