

Characterisation of major and minor *Dermatophagoides* allergens in canine atopic dermatitis

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SUMMARY

Atopic dermatitis is a well-recognised chronic inflammatory skin disease of humans and dogs. Most atopic dogs are sensitised to *Dermatophagoides* mites. The aim of this study was to characterise allergens in different *Dermatophagoides* species using polyclonal and monoclonal antibodies to canine IgE. Western blots were prepared from crude extracts of *D. farinae*, *D. pteronyssinus* and *D. microceras*, and purified group 1 and 2 allergens under reducing and non-reducing conditions. They were probed with sera from atopic (n=33) and healthy (n=27) dogs. There was no significant difference in the sensitivity or specificity between the polyclonal and monoclonal sera in detecting *Dermatophagoides*-specific IgE. Major allergens common to both *D. farinae* and *D. pteronyssinus* were detected at 97–98 kDa, 103–104 kDa and 134–139 kDa on both reducing and non-reducing blots. Major allergens at 84–85 kDa, 65–69 kDa and 44–45 kDa were only recognised on reducing blots, suggesting that these are fragments of the larger allergens. Only a few sera recognised group 1 or 2 allergens on blots of crude extracts or purified allergens. These results confirm that, in atopic dogs, high molecular weight allergens are the most important *Dermatophagoides* allergens, rather than the low molecular weight group 1 and 2 proteins. © 2001 Harcourt Publishers Ltd

ATOPIC dermatitis (AD) is a well-recognised chronic inflammatory skin disease of humans and dogs (Rothe and Grant-Kels 1996, Scott et al 1995), characterised by cutaneous and circulating IgE specific for various environmental allergens (Day et al 1999, Leung 2000). The most important allergens in both human and canine AD are house dust mites (HDM) (Halbert et al 1995, Sture et al 1995). *Dermatophagoides pteronyssinus* is the most common in the UK, although *D. farinae* is also found (Thomas et al 1998). A third species, *D. microceras*, is rare in the UK (O'Hehir 1988).

The most important HDM allergens in human AD are the group 1 and group 2 proteins, Der p1 and 2 from *D. pteronyssinus*, and Der f1 and 2 from *D. farinae* (Thomas et al 1998). High IgE titres to group 1 and 2 allergens are present in 70–80 per cent of atopic human sera, whereas variable titres to other allergens are found in only 40–50 per cent of sera (Le Mao et al 1998, Thomas et al 1998).

The identification of HDM allergens in canine AD is controversial. Studies using anti-canine IgGd (Noli et al 1996) and recombinant FcεRIα (McCall et al 2000, Shaw 2000) detected major allergens of 60–70 kDa and 90–109 kDa on Western blots of *D. farinae* and *D. pteronyssinus*. However, although 69 per cent of atopic sera also recognised Der f1 using FcεRIα in one study (McCall et al 1999), others detected very little binding to

group 1 and 2 allergens using either FcεRIα (Shaw 2000) or anti-canine IgGd (Noli et al 1996). In contrast, the only study using anti-canine IgE (in ELISAs and dot blots) reported that 50 per cent of atopic sera recognised both Der p1 and 2, and Der f1 and 2 (Masuda et al 1999).

These conflicting results could arise from the variable allergen content of different extracts (Le Mao et al 1998) and specificity and sensitivity of different antisera (Steward and Male 1998). Furthermore, gel separation of protein extracts under reducing conditions (Laemmli 1970) can denature IgE epitopes on Der p1 and 2 (Lombardero et al 1990).

The aim of this study, therefore, was to characterise further and compare *D. farinae* and *D. pteronyssinus* allergens on reducing and non-reducing Western blots of different HDM extracts using both monoclonal and polyclonal antibodies to canine IgE.

MATERIALS AND METHODS

Study population

Dogs with AD were recruited from the dermatology clinic at the University of Edinburgh Hospital for Small Animals. Diagnosis was based on compatible history and clinical signs, and exclusion of other pruritic dermatoses (Willemse 1986). Coat brushings, skin scrapes and trial therapy were used to eliminate ectoparasites. All dogs underwent a 6-week, home-cooked diet trial to eliminate food intolerance. Seborrhoea, pyoderma and *Malassezia* were managed appropriately.

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No anti-inflammatory medication was given for at least 3 weeks prior to examination.

All dogs with a clinical diagnosis of AD were skin-tested with 57 allergens, including *D. farinae* and *D. pteronyssinus* (Greer Laboratories, Lenoir, USA). Dogs were sedated with 0.15 mg kg⁻¹ xylazine IM. Hair was clipped from the lateral flank and 0.05 ml of each allergen extract, 1/100,000 w/v histamine (positive control) and diluent (negative control) were injected intradermally. After 20 minutes, test-sites were scored from 0 to 4 compared to the controls. Reactions ≥ 2 were considered positive (Nuttall et al 1998).

Control samples were taken from healthy dogs presented for euthanasia at the University of Edinburgh Hospital for Small Animals with no history and clinical signs of pruritus or conditions likely to alter immune function.

Sample collection

Blood samples from dogs with AD (n = 33) were collected by jugular venepuncture. Blood samples from healthy dogs (n = 27) were collected by cardiac puncture immediately after euthanasia. Serum was separated by centrifugation and stored at -20 °C.

Allergen extracts

Freeze-dried HDM extracts were obtained from a number of sources (see Table 1). Each extract was reconstituted in sterile phosphate buffered saline at 1 mg ml⁻¹ (BCA protein assay; Pierce Laboratories, Rockford, USA). 100 µl aliquots were stored at -20 °C.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Ten microlitres of each extract was diluted 1:1 in either reducing (0.125 M tris/HCl, 4 per cent SDS, 20 per

cent glycerol, 0.005 per cent bromophenol blue and 10 per cent 2-mercaptoethanol) or non-reducing (without 2-mercaptoethanol) loading buffer. SDS-PAGE was performed on 10 per cent tris-glycine resolving gels according to the manufacturers recommendations (Mini-Protein II®; Biorad, Hercules, USA). Duplicate gels were stained with 2.5 per cent Coomassie blue and silver, transilluminated with white light and photographed (The Imager; Appligene Oncor, Chester-le-Street, UK).

Western blots

SDS-PAGE was performed using a single well across the width of the gel with 200 µg of the *D. farinae*, *D. pteronyssinus* and *D. microceras* extracts, and 50 µg of the purified group 1 and 2 extracts in reducing and non-reducing loading buffer. Proteins were transferred to PVDF membranes (Immobilon-P; Millipore, Bedford, USA) using 0.3 M tris-HCl/20 per cent methanol (pH 10.4) lower anode, 25 mM tris-HCl/20 per cent methanol (pH 10.4) upper anode and 25 mM tris-HCl/40 mM 6-amino-n-hexonic acid/20 per cent methanol (pH 9.4) cathode buffers according to the manufacturers instructions (Transblot SD; Biorad). Membranes were dried and stored at 4 °C.

Immunoblotting

The molecular weight markers (Biorad broad range) and a strip to identify protein bands were removed and stained with 2.5 per cent Coomassie blue.

The membranes were blocked in 5 per cent skimmed milk/tris-buffered saline (TBS) for 1 hour at room temperature, briefly rinsed in TBS, then placed in an MN28 Miniblotter (Immunectics, Cambridge, USA). Appropriate channels were incubated for 1 hour at room temperature with 85 µl of a 1/5 dilution of serum, then 85 µl of either a 1/500 dilution of mouse monoclonal (D9#31116; D. J. Deboer, University of Wisconsin) (Mab) or a 1/100 dilution of goat polyclonal (A40-125; Bethyl Laboratories, Montgomery, USA) (Pab) anti-canine IgE. Blanks and control channels with no serum or no anti-canine IgE were included in each assay. Channels were washed with 10 ml of 0.05 per cent tween 20/TBS (TTBS) after each step. The miniblotter was then dismantled and the membranes incubated at room temperature for 1 hour with 20 ml of horseradish peroxidase-conjugated bovine monoclonal anti-mouse IgG (MCA1421; Serotec, Kidlington, UK) or mouse monoclonal anti-goat IgG (GT-34; Sigma, Poole, UK) diluted 1/1000 and 1/5000 respectively. The membranes were washed 3 times for 5 minutes in TTBS, then twice for 5 minutes in TBS. The protein surface of each membrane was covered with luminol solution (ECL; Amersham-Pharmacia Biotech, Little Chalfont, UK) for 1 minute and each blot imaged (IS440CF; Kodak, Rochester, USA).

All reagents were diluted in 1 per cent skimmed milk-TBS. Optimum conditions and reproducibility were established prior to the study (data not shown).

TABLE 1: Source of house dust mite extracts

Crude extracts	
Antigen extract	Source
<i>Dermatophagoides farinae</i>	Greer ALK
<i>D. pteronyssinus</i>	Greer ALK AP Rayne
<i>D. microceras</i>	ALK
Purified extracts	
Antigen extract	Source
Der p1	AP Rayne
Der f1	ALK
Der p2	AP
Der f2	ALK

Greer — Greer Laboratories, Lenoir, USA; ALK — ALK-Abello, Hørsholm Denmark; AP — Allergo-Pharma, Hamburg, Germany; Rayne — Rayne Laboratory, University of Edinburgh, UK.

Data analysis

Four replicates of each allergen blot were generated for both atopic and healthy sera-reducing buffer with Mab and Pab, and non-reducing buffer with Mab and Pab. The numbers of positive sera identified on the four replicate blots were compared using Fisher's exact test (Instat[®]; Graphpad Inc., San Diego, USA). Mann-Whitney tests were used to compare the numbers of positive atopic and healthy sera that recognised each allergen extract (Instat[®]; Graphpad Inc., San Diego, USA). Significance was set at $P < 0.05$ for each test.

Standard curves constructed using the molecular weight markers were used to calculate the mean molecular weight of allergens recognised by each serum sample (MS Excel 2000; Microsoft Corp., Seattle, USA). Major allergens were defined as those recognised by 50 per cent or more of positive sera.

RESULTS

Intradermal skin tests

Most dogs with AD had positive reactions to *D farinae* and *D pteronyssinus* (Table 2). Two dogs had reactions to *D farinae* but not to *D pteronyssinus*. Occasional reactions were seen to other mites, insects, pollens and moulds. Four dogs with AD had no positive reactions.

SDS-PAGE of HDM extracts

The protein content of the allergen extracts varied widely (Fig 1). In particular, only a few low molecular weight proteins were detected on the ALK *D farinae*, ALK and Rayne *D pteronyssinus*, and ALK *D microceras* gels. The appropriate bands were observed on the purified allergen gels. Non-reducing gels were similar, though not identical to the corresponding reducing gels. There were few differences between gels stained with silver and Coomassie blue (data not shown).

Immunoblotting

There was no non-specific binding by the anti-canine IgE antibodies or secondary conjugates. Abolishing binding by pre-heating serum at 56°C for 1 hour confirmed the specificity of both antibodies for IgE (Tizard 1996). One serum sample from a skin-test negative dog detected *Dermatophagoides* allergens under all blotting conditions and three samples detected allergens on individual blots.

As there were no significant differences in the number of positive sera identified using either Pab or Mab for any of the reducing or non-reducing blots

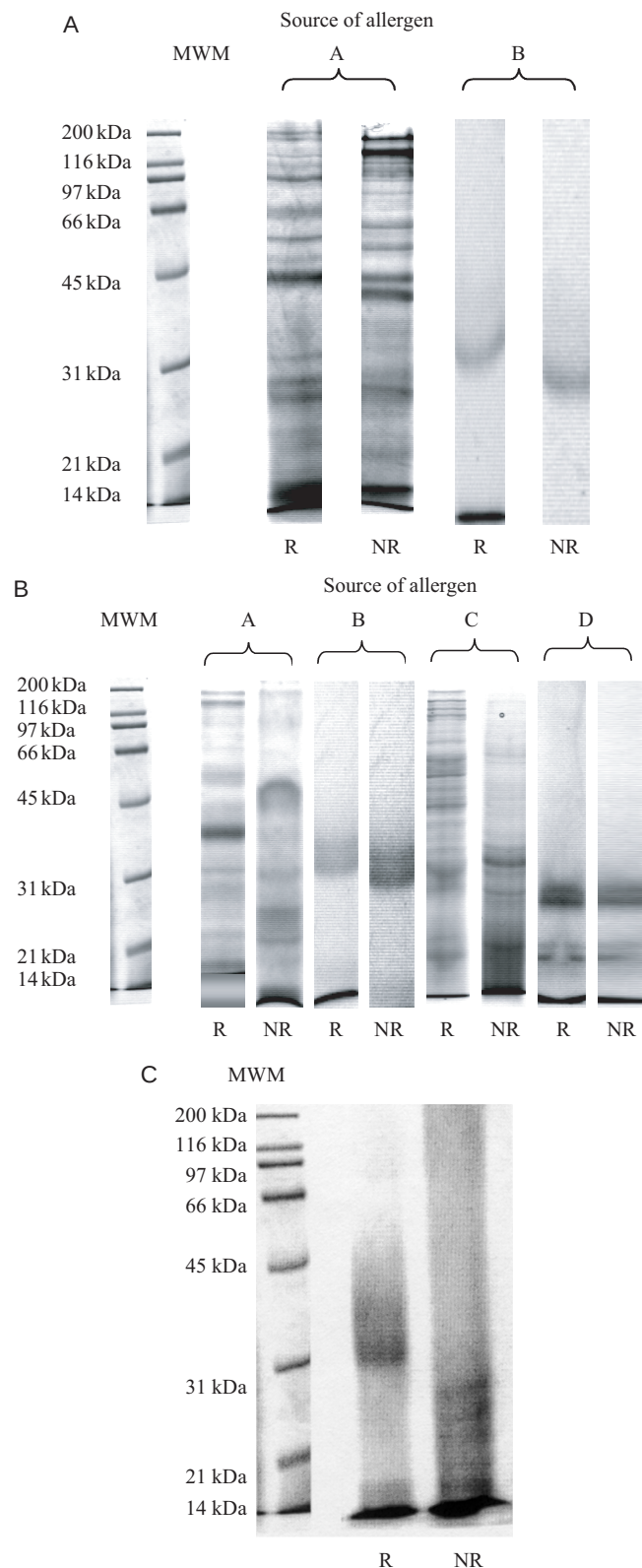


TABLE 2: Intradermal skin test reactions to *Dermatophagoides farinae* and *D pteronyssinus* in dogs with atopic dermatitis (n = 33)

Allergen extract	Positive	Negative
<i>Dermatophagoides farinae</i>	24	9
<i>D pteronyssinus</i>	22	11

FIG 1: SDS-PAGE of crude and purified *Dermatophagoides* extracts (2.5 per cent Coomassie blue stain) A – *D farinae*; B – *D pteronyssinus*; C – *D microceras*; D – Group 1 allergens; E – Group 2 allergens; MWM – Molecular weight markers (broad range; Biorad, Hercules, USA); R – Reducing buffer; NR – Non-reducing buffer; Source of allergens: A – Greer Laboratories, Lenoir, USA; B – ALK-Abéllo, Hørsholm, Denmark; C – Allergo-Pharma, Hamburg, Germany; D – Rayne Laboratory, University of Edinburgh, UK.

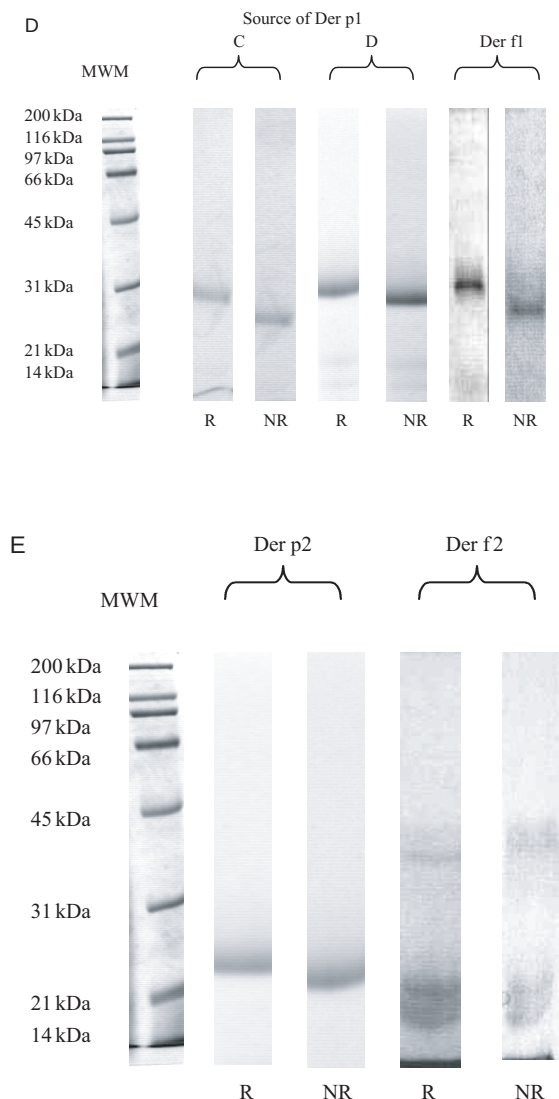


FIG1: Continued.

(Table 3), the results were combined to give four replicates (Mab/reducing blot, Mab/non-reducing blot, Pab antisera/reducing blot and Pab/non-reducing blot) for each allergen extract in subsequent analyses.

IgE binding to D farinae extracts

Significantly more atopic than healthy sera recognised *D farinae* allergens ($P < 0.05$), although more atopic sera bound to the Greer compared to the ALK extract ($P < 0.05$) (Fig 2A).

IgE binding to the D pteronyssinus extracts

Significantly more atopic than healthy sera bound to the Greer, ALK and AP *D pteronyssinus* extracts ($P < 0.05$) (Fig 2B). Far fewer sera recognised the Rayne extract compared to the others ($P < 0.05$). This is prepared from mite media and largely consists of Der p1 and Der p2 (G. Hoyne, University of Edinburgh, personal communication). Although more atopic sera bound to the Greer *D farinae* extract compared to the *D pteronyssinus* extract ($P < 0.05$), there was no difference between the ALK extracts.

IgE binding to the D microceras extract

Significantly fewer atopic and healthy sera (range 4–21 per cent) recognised the ALK *D microceras* extract compared to the ALK *D farinae* or *D pteronyssinus* extracts ($P < 0.05$).

IgE binding to the purified Group 1 and 2 allergens

Apart from high molecular weight contaminants on the ALK Der f1 and Der f2 blots (range 8–29 per cent), very few sera recognised any of the purified group 1 and 2 allergens (range 0–4 per cent).

TABLE 3: Percentage of atopic (n = 33) and healthy (n = 27) sera that bound to Western blots of crude and purified house dust mite allergens

Allergen extract	Atopic sera				Healthy sera			
	Reducing blot		Non-reducing blot		Reducing blot		Non-reducing blot	
	Mab	Pab	Mab	Pab	Mab	Pab	Mab	Pab
Greer DF	67	58	52	58	23	12	15	11
ALK DF	50	32	25	42	15	15	4	8
Greer DP	50	35	50	24	8	32	4	4
ALK DP	30	26	28	32	19	4	15	22
APDP	48	48	32	36	4	7	16	16
Rayne DP	7	0	0	0	4	0	0	0
ALKDM	21	8	4	4	4	7	4	7
AP Der p1	0	0	0	0	0	0	0	0
Rayne Der p1	0	0	0	4	0	0	4	0
AP Der p2	0	0	0	0	0	0	0	0
ALK Der f1	12	8	29	12	0	0	0	0
ALK Der f2	12	20	15	11	0	0	0	0

DF – *Dermatophagoides farinae*; DP – *D pteronyssinus*; DM – *D microceras*; Greer – Greer Laboratories, Lenoir, USA; ALK – ALK-Ab  llo, H  rsholm Denmark; AP – Allergo-Pharma, Hamburg, Germany; Rayne – Rayne Laboratory, University of Edinburgh, UK; Mab – monoclonal anti-canine IgE; Pab – polyclonal anti-canine IgE.

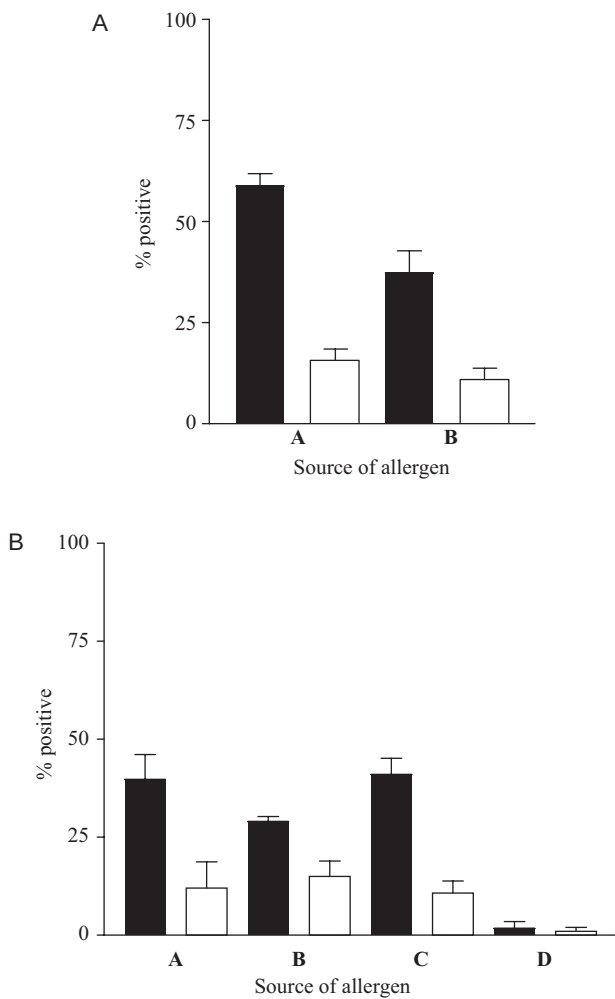


FIG 2: Percentage of atopic and healthy sera that bound to Western blots of *Dermatophagoides* allergens A – *D. farinae*; B – *D. pteronyssinus*; Bar – sem; ■ Atopic sera; □ Healthy sera.

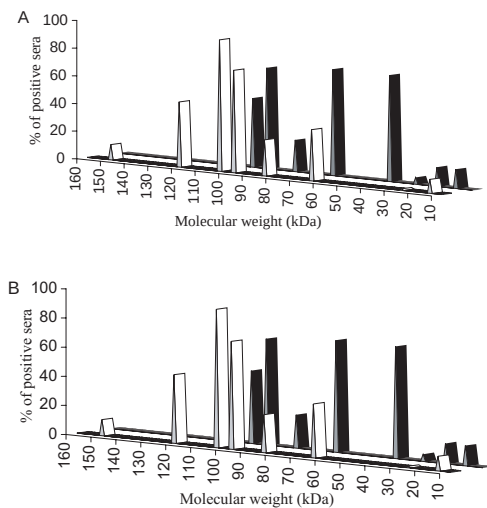


FIG 3: Major and minor allergens in the Greer *Dermatophagoides* extracts (bars greater than 50 per cent indicate major allergens). A – *D. farinae*; B – *D. pteronyssinus*; ■ – Atopic sera; ■ – Healthy sera.

TABLE 4: Major and minor allergens identified in *D. farinae* and *D. pteronyssinus*

Major allergens (kDa)		Minor allergens (kDa)	
Reducing blot	Non-reducing blot	Reducing blot	Non-reducing blot
a. <i>D. farinae</i>			
151	139	116	151
134	103	65	120
104	98	32	63
97		25	28
84		14	21
44			
b. <i>D. pteronyssinus</i>			
103	104	115	150
97	98	85	121
69		34	85
45		25	65
		17	27
			15

Major and minor allergens

Major allergens of 97–98 kDa and 103–104 kDa were common to reducing and non-reducing blots of *D. farinae* and *D. pteronyssinus*. Major allergens under 97 kDa were only seen on reducing blots. A range of minor allergens from 15–150 kDa was identified on both reducing and non-reducing blots of *D. farinae* and *D. pteronyssinus* (Fig 3, Table 4).

DISCUSSION

This study has, for the first time, characterised in detail the recognition of major and minor *Dermatophagoides* allergens by atopic sera using anti-canine IgE. Major allergens common to *D. farinae* and *D. pteronyssinus* were detected as double bands at 97–98 kDa and 103–104 kDa on both reducing and non-reducing blots. These probably represent the double band previously reported as a major allergen using anti-canine IgGd and FcεRIα (Noli et al 1996, McCall et al 2000, Shaw 2000). This was identified as a chitinase, Der f15 (McCall et al 2000), although a 98 kDa allergen recognised by human sera, Der f11, is homologous to a paramyosin (Tsai et al 1999). Unidentified major *D. farinae* allergens of 95–101 kDa are also recognised by human sera (Le Mao et al 1998). It is as yet unclear whether canine and human atopic sera recognise similar high molecular weight allergens.

The *D. farinae* major allergen detected at 134–139 kDa in this study has no obvious homologue, but may represent an alternately glycosylated form of the 97–104 kDa allergen, whose native structure is approximately 63 kDa (McCall et al 2000). Other major allergens at 84–85 kDa, 65–69 kDa and 44–45 kDa were only recognised on reducing blots, suggesting these are denatured forms of the larger allergens. Degradation products have also been identified on *D. farinae* blots probed with human sera (Le Mao et al 1998). Results from reducing blots should be therefore interpreted with care. However, despite the fact that reducing

agents can alter protein structure and denature IgE epitopes (Lombardero et al 1990), there were no significant differences in the numbers of sera that bound to reducing and non-reducing blots of each allergen. Similarly there was no difference in sensitivity or specificity between polyclonal and monoclonal anti-canine IgE, suggesting all the techniques are equally sensitive and specific. Our results show that, in contrast to humans, group 1 and 2 *Dermatophagoides* allergens are of limited relevance to canine atopic dermatitis. No positive control canine sera to these allergens were available, but protein bands were identified on each blot and they had been used in human and murine studies (G. Nordskov Hansen, ALK-Abello; O. Cromwell, Allergo-Pharma; N. Savage, University of Edinburgh; personal communications). Although not visible on the gels, a few atopic sera recognised trace amounts of the 97–104 kDa major allergens on the Der f1 and 2 blots. This may explain the high frequency of binding of canine atopic sera to group 1 and 2 allergens in dot blots and ELISAs (Masuda et al 1999), where individual proteins are not separated.

To our knowledge, this is the first study using anti-canine IgE to demonstrate that different *Dermatophagoides* allergens are important in human and canine AD. This is surprising, as many allergens have intrinsic allergenic properties (Huby et al 2000). In particular, Der p1 degrades tight junctions, cleaves α 1-antitrypsin, CD23 and CD25, which directly activates mast cells and basophils, inhibits T_{H1} differentiation and increases epithelial permeability, protease activity, IgE and inflammatory mediator release (Schulz et al 1999, Shakib and Gough 2000, Wan et al 2000). The 98 kDa chitinase is glycosylated, which enhances IgE binding and uptake by antigen presenting cells (APCs) (Huby et al 2000), but it is unknown if any of the allergens identified with canine sera are biologically active. In vivo activity could also be substrate and species specific. Furthermore, unlike B-cells, T-cells recognise short sequences of 10–15 amino acids associated with class II MHC molecules (Renz 1995). These are derived from allergen processed by APCs (Carrasco-Marín et al 1998), which profoundly influence the subsequent immune responses (Maekawa et al 1998). If canine APCs present a different array of peptides or if T-cell receptor and MHC class II repertoires are very distinct from humans, the specificity of allergen recognition could also differ.

Far fewer sera recognised *D. microceras* compared to *D. farinae* and *D. pteronyssinus*. This study did not discriminate between sensitisation to individual *D. farinae* and *D. pteronyssinus* allergens or cross-reacting allergens specific to these mites. However, *D. farinae* and *D. pteronyssinus* allergens are 80–90 per cent homologous (Thomas et al 1998) and cross-inhibition with canine sera has shown that their allergens strongly cross-react (Masuda et al 1999).

Recognition of the *D. farinae* extracts by atopic sera was influenced by the allergen source. However, there was no difference in binding between the *D. pteronyssinus* extracts, despite a similar variation in protein content. Moreover, differences in binding between *D. farinae* and *D. pteronyssinus* were restricted

to a single allergen source. The concentration of key allergens in certain extracts could explain why *D. farinae*, despite being less common than *D. pteronyssinus* in the UK (Thomas et al 1998), is a more prominent allergen in canine AD (Sture et al 1995). However, a recent study speculated that an apparently low prevalence of *D. farinae* could be due to seasonal fluctuations, as equal levels of Der f1 and Der p1 were present in house dust (Sopelete et al 2000). Further work to define and standardise the allergen content of *Dermatophagoides* extracts is needed.

There was good correlation between skin tests and immunoblotting for *D. pteronyssinus* and *D. farinae*, although immunoblots were less sensitive. Epitopes may be less available after gel separation and blotting or blocked by allergen specific IgG, which parallels allergen specific IgE in canine sera (Day et al 1999). The few positive immunoblots from skin-test negative dogs may be due to technical problems, but allergen specific IgE also occurs in healthy and skin-test negative atopic dogs (Day et al 1999).

In conclusion, this study has shown that major *Dermatophagoides* allergens in canine AD are restricted to proteins greater than 95 kDa. Group 1 and 2 allergens are of limited importance. Using standardised allergens will be important in the future diagnosis and treatment of AD.

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