

Allergen-specific IgE and IgGd antibodies in atopic and normal dogs

Tan Mei Lian, Richard E.W. Halliwell*

*Department of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Studies,
University of Edinburgh, Summerhall Square, Edinburgh EH9 1QH, Scotland, UK*

Received 12 February 1998; accepted 7 August 1998

Abstract

Intradermal skin tests (IDSTs) were performed on 65 atopic and 24 normal dogs. The levels of allergen-specific IgE and IgGd antibodies were determined in serum samples by enzyme-linked immunosorbent assay (ELISA) using the same 12 allergens that were used in the IDST on normal dogs. The correlation between the levels of IgE and IgGd to *Dermatophagoides farinae* (DF) and *Dermatophagoides pteronyssinus* (DP) was examined. The sensitivity, specificity and positive and negative predictive values of allergen-specific IgE and IgGd levels in the total dog population were also compared. Results were consistent and reproducible for 9/12 allergens, but in the case of house dust, flea and *Alternaria tenuis*, a less discriminating standard curve and the fact that the negative control gave positive results, suggests non-specific binding and that these allergens are complex and should not be employed without further purification. A high percentage of atopic dogs had positive IDSTs and detectable IgE and IgGd antibodies to DF, DP and house dust. Similar results were obtained in the normal dog population. There were significant correlations between allergen-specific IgE and IgGd levels to DF and DP. However, in contrast to IgE, allergen-specific IgGd in normal dogs was higher than in atopic dogs. Furthermore, a high percentage of the atopic population had detectable IgGd to unrelated allergens, despite negative IDSTs. Overall, the negative predictive values were similar for both IgE and IgGd. Sensitivities were higher in the allergen-specific IgGd assays, but the specificities and positive predictive values were higher in the allergen-specific IgE assays. In conclusion, the concordance of IDSTs with ELISA results to DF and DP in normal dogs without clinical signs implied the possible heterogeneity of IgE in dogs. The presence of IgGd directed against apparently irrelevant allergens in atopic patients and the high levels of IgGd in normal dogs to the most common allergens, DF and DP, implied an uncertain role of IgGd in canine atopic disease. Therefore, the detection of allergen-specific IgE is a more useful adjunct to the diagnosis of atopic disease in the dog than IgGd. © 1998 Elsevier Science B.V. All rights reserved.

* Corresponding author. Tel.: +44+131+650+6294; fax: +44+131+650+6588.

Keywords: Immunoglobulin E (IgE); Immunoglobulin Gd (IgGd); Enzyme-linked immunosorbent assay (ELISA); Intradermal skin test (IDST); Dog

1. Introduction

Atopic disease in dogs is defined as a hereditary, IgE-mediated hypersensitivity to environmental allergens (Halliwell and Gorman, 1989). The generally accepted criteria for diagnosis include compatible history and clinical signs, together with evidence of allergen-specific IgE assessed by either intradermal or serological tests (Halliwell, 1990). However, most dermatologists favour intradermal skin tests (IDSTs) because they have been performed routinely for approximately 2 decades and the indications and limitations are well known. Furthermore, the specificity of commercially available test kits using polyclonal anticanine IgE has been questioned (Codner and Lessard, 1993; Bond et al., 1994).

In man, the association of IgE with hypersensitivity disorders is well accepted. However, the association of IgG4 with such diseases is more controversial and paradoxical. This subclass may act as an inefficient anaphylactic antibody that initiates immediate hypersensitivity. However, it may also act as a 'blocking antibody' that might prevent the binding of IgE to allergen (Aalberse et al., 1993). Most studies have revealed that the IgG4 response is closely correlated with that of IgE (Shakib et al., 1977; Desvaux et al., 1989; Lucas, 1990).

The role of IgG antibody in canine atopic disease is less well defined. Willemse et al. (1985a, b) identified a non-IgE anaphylactic antibody, termed IgGd, in the sera of atopic dogs. It has been proposed that IgGd may be important in the pathogenesis of atopic disease and a possible source of discordant results between skin tests and in vitro tests for IgE antibodies in some atopic dogs.

Hites et al. (1989) showed that allergen-specific IgG was detectable in normal and atopic dogs, but levels were highest in atopic dogs receiving immunotherapy. Wheeler (1993) found detectable levels of grass pollen-specific IgG antibody in normal dogs whose sera showed negative passive cutaneous anaphylaxis (PCA) tests. Hill et al. (1995) observed significantly higher levels of total IgG in atopic dogs than in normal dogs. However, in neither of these studies were subclasses of IgG investigated.

Recently, Day et al. (1996) showed that the serum levels of allergen-specific IgE and IgG in atopic dogs were significantly correlated. In addition, using a panel of newly developed monoclonal antibodies directed against four subclasses of canine IgG (Mazza et al., 1994), the IgG response to *Dermatophagoides farinae* (DF) and *Dermatophagoides pteronyssinus* (DP) was found to be dominated by the IgG4 subclass. However, the relationship between the newly redefined IgG subclasses and the classically defined IgG subclasses is unclear and it has also been demonstrated that commercially available anticanine IgG subclass-specific antisera were not specific for any of the four newly defined subclasses (Mazza et al., 1993).

The aims of this study were to determine the levels of allergen-specific IgE and IgGd in atopic and normal dogs by the means of enzyme-linked immunosorbent assay (ELISA) and to examine the correlation between the two isotypes. In addition, assuming the

IDST as the ‘Gold Standard,’ the sensitivity, specificity, positive and negative predictive values of allergen-specific IgE and IgGd levels in atopic and normal dogs were assessed.

2. Materials and methods

2.1. Animals

2.1.1. Atopic dogs

Atopic disease was diagnosed in 59 dogs referred to the Royal (Dick) School of Veterinary Studies (R(D)SVS), University of Edinburgh, and six dogs to Godiva Referrals, Coventry. All were kept under household conditions. A variety of breeds were included, ranging in age from 9 months to 10 years (mean 4.3 years). Females represented 30 of the cases (9 were neutered). Four of the 35 males were castrated.

The diagnosis of atopic disease was made by a combination of compatible historical evidence, clinical signs, lack of response to 6 weeks dietary restriction and absence of any other dermatological disease that could account for the clinical signs, together with the presence of at least one strongly positive IDST.

2.1.2. Normal dogs

Twenty-four privately owned physically healthy adult dogs without any present or prior history of skin problems were selected as the control population. All were ectoparasite-free as assessed by visual inspection. Except for two working Border collies, they were kept under household conditions. Their ages ranged from 8 months to 16 years (mean 5.0 years). Fourteen were bitches (9 were neutered) and 3 were castrated males. Four were of mixed breeding while the remainder represented 8 different breeds.

2.2. Performance of the IDST

2.2.1. Allergens employed

Fifty-seven allergens were used in the IDST for the atopic population and 12 of them were selected for the normal dog population. These 12 were from six different groups, and included those to which positive IDSTs are most commonly seen in atopic dogs in the UK (Sture et al., 1995) (Table 1). The majority of the stock extracts including all the allergens used for the in vitro studies were obtained from a single commercial allergy laboratory (Greer Laboratories, Lenoir, NC). The remaining were obtained from ARTU (Artuvetrin Allergens; ARTU Biologicals, Lelystad, The Netherlands). Allergens were individually diluted to appropriate dilutions which were believed to be non-irritant based on the previous published work (August, 1982) and experience in the R(D)SVS Dermatology Clinic.

Thirty-three of the allergens used at Coventry were purchased from ARTU and used at the manufacturer’s recommended strengths. The other 12 were the same as used in the IDST on normal dogs at the R(D)SVS.

Table 1

Allergens employed in the intradermal skin test for both atopic and normal dogs and used in ELISA

Number	Allergens	Concentration supplied	Units	Concentration used for skin tests	Units	Source
<i>Mites/dusts</i>						
1	<i>Dermatophagoides pteronyssinus</i>	1 : 100	w/v	1 : 10000	w/v	Greer
2	<i>Dermatophagoides farinae</i>	1 : 100	w/v	1 : 10000	w/v	Greer
3	House dust	20 000	PNU	1000	PNU	Greer
<i>Insects</i>						
4	Flea	1 : 100	w/v	1000	w/v	Greer
<i>Trees</i>						
5	Tag alder	20 000	PNU	1000	PNU	Greer
6	American beech	20 000	PNU	1000	PNU	Greer
<i>Weeds</i>						
7	Yellow dock	20 000	PNU	1000	PNU	Greer
8	Common mugwort	20 000	PNU	1000	PNU	Greer
<i>Grasses</i>						
9	Kentucky blue	20 000	PNU	1000	PNU	Greer
10	Meadow fescue	20 000	PNU	1000	PNU	Greer
11	Timothy	20 000	PNU	1000	PNU	Greer
<i>Moulds/smuts</i>						
12	<i>Alternaria tenuis</i>	20 000	PNU	1000	PNU	Greer

2.2.2. Skin testing protocol

Routine withdrawal periods for glucocorticoids and antihistamines were observed in all the cases. Dogs were sedated with 0.04 mg kg⁻¹ of atropine subcutaneously and 0.5 mg kg⁻¹ of xylazine hydrochloride (Virbaxyl 2%, Virbac, Cambridge, UK) intramuscularly. Hair on the lateral aspect of the thorax was clipped and 0.05 ml of each allergen solution was injected intradermally in a standardised pattern, together with a positive (1/100 000 w/v histamine phosphate) and negative (diluent) control.

Sites were examined 15–20 min after injection with the aid of incident light. Skin tests were graded using a +1–+4 scale based on erythema, turgidity, elevation of the wheal and wheal size (Halliwell and Gorman, 1989). Grades were assigned by comparing the wheal response to each of the tested allergens with negative and positive controls. A grade of +1 was just discernibly greater than the negative control, and a +4 approximated the size of the histamine wheal.

2.3. In vitro ELISA

2.3.1. Serum samples

Blood was drawn by venipuncture, allowed to clot at room temperature and centrifuged at 3000 rpm for 10–20 min at 4°C. Serum samples were stored at –20°C.

2.3.2. Preparation of antisera

Rabbit anticanine IgE was prepared as described earlier (Halliwell and Longino, 1985). The specificity of the batch of anti-IgE was checked by assaying the positive control serum before and after heating for 4 h at 56°C.

Canine IgG_{2ab} was prepared from normal canine serum by precipitation with 33.33% saturated ammonium sulphate followed by exclusion from diethylaminoethyl cellulose (DEAE) (DE52; Whatman, Maidstone, Kent, UK) using 0.01 M phosphate buffer, pH 8.0. Canine IgGd was isolated from a myeloma serum (Schwartzman, 1984) by gradient elution on DEAE.

Anticanine IgGd was prepared by immunizing rabbits with three bi-weekly injections of 1 mg aliquots of purified IgGd in incomplete Freund's adjuvant. The resulting antiserum was rendered specific for IgGd by passage through a CNBr-activated column of Sepharose 4B (Sigma, Poole, UK) coupled to IgG_{2a,b}. Purified antibody was then prepared by absorption and elution from a column of Sepharose 4B coupled to a 40% ammonium sulphate precipitate of normal canine serum. The eluate was specific for IgGd as assessed by immunoelectrophoresis with the presence of a single precipitation band with γ 1 electrophoretic mobility (Halliwell et al., 1972).

Purified rabbit anti-canine antibody was coupled to alkaline phosphatase by a modified gluteraldehyde procedure (Engvall and Perlmann, 1972).

2.3.3. ELISA

A direct ELISA was employed. All allergens were obtained from Greer Laboratories (Lenoir, NC), and were the same as those used in the IDST on normal and atopic dogs.

Prior to the commencement of the assays, sera were identified which had high levels of allergen-specific antibody for each antibody class, and for each allergen. These sera were given an arbitrary relative antibody unit (RAU) of 10 000 when undiluted (Halliwell et al., 1993). Doubling dilutions of the reference serum were prepared in ELISA buffer (0.1% human serum albumin (HSA) (essentially globulin-free) (Sigma, Poole, UK) in washing buffer) to generate a standard curve which was included in each plate. Serum from a 6-year-old female laboratory beagle was used as a negative control serum in each assay. The beagle was presumably never exposed to house dust mite allergen and gave negative skin tests to the panel of 57 allergens.

(1) *Procedures*: Wells of polystyrene microtitre plates (Immulon 4, Dynatech, Billingshurst, UK) were coated with 200 μ l of the allergen diluted in a carbonate/bicarbonate buffer (pH 9.6). The dilution of the concentrated allergen preparation that was found to be optimal was, 1/100 for mites, insect and mould allergens and 1/200 for trees, weeds and grass pollen. The plates were incubated overnight in a humidity chamber at 37°C. After rinsing the plates six times with a washing buffer (PBS pH 7.2, 0.05% Tween 20 (Sigma)), the reactive sites were blocked by the addition of 250 μ l of 0.5% HSA in a washing buffer and overnight incubation at 4°C.

After further rinsing, 100 μ l of test serum was added to wells in duplicate, along with diluent buffer blanks and a negative control serum. The plates were then incubated for 2 h at 37°C. After a further six washes, 100 μ l of alkaline phosphatase-conjugated rabbit anticanine IgE (1 : 25 dilution) or IgGd (1 : 100 in the case of mite, insect and mould antigens, 1 : 200 for pollen antigens), respectively, was added. Plates assayed for

allergen-specific IgGd were incubated for 1 h 30 min at 37°C, whereas plates assayed for allergen-specific IgE were incubated for 24 h at 4°C.

Following further washing as previously done, 100 µl of the substrate was added to each well, which was *p*-nitrophenyl phosphate disodium hexahydrate (Sigma) in substrate buffer (0.01% MgCl₂, 9.7% diethanolamine and 0.02% NaN₃ adjusted to pH 9.8 with HCl). The plates were left to develop in the dark at 37°C and read every 15 min until the optical density (OD) at 405 nm of the lowest dilutions of the standard curve was >1.0. The resultant colour change was measured by the use of a programmable automated ELISA reader (MR 5000, Dynatech, Billingshurst, UK) with subtraction of blank well values. The standard curve was generated by plotting the OD against log₁₀ reference serum concentration.

Initially, all the sera were assayed at 1/10 dilution in ELISA buffer. The assays were then repeated at a higher dilution if the reading did not lie within the sensitive part of the standard curve. A serum whose OD fell below the end point of the standard curve (EPSC) was deemed to be negative. For the purposes of statistical analyses, these sera were assigned a value half way between the RAU of the EPSC and zero.

(2) *Reproducibility of the ELISA*: Eight replicates of the mid-point dilution of reference sera were assayed on three plates on different days to obtain the intra-assay and inter-assay coefficients of variations (CV).

(3) *Determination of sensitivity, specificity and positive and negative predictive values*: Assuming the IDST as the 'Gold Standard' for the diagnosis of atopic disease, the sensitivity, specificity and predictive values of positive and negative results of the total population ($n = 89$) were examined for IgE and for IgGd for all the allergens. Clearly, the cut-off point is critical in this determination, and so the assessment was made using a cut-off point of the EPSC and the above parameters were assessed using a standard formula (Gerstman and Cappucci, 1986).

2.4. Statistical analyses

The median level of allergen-specific IgE and IgGd of the atopic and normal dog populations were compared by an unpaired non-parametric Mann–Whitney analysis. Correlations between the levels of IgE and IgGd were calculated using a Spearman's rank correlation and the correlation between the allergen-specific IgE and IgGd levels and skin test reactions were compared by Kruskal–Wallis one-way analyses of variance (ANOVA). The values were considered statistically significantly different if the probability (p) was less than 0.05.

3. Results

3.1. Intradermal skin testing

3.1.1. Atopic dogs

At least one positive reaction was obtained in 54 out of the 57 allergens employed. Of the 65 atopic dogs, 58 (89.2%) were skin test-positive to *Dermatophagoides farinae* (DF),

56 (86.2%) to house dust and 45 (69.2%) to *Dermatophagoides pteronyssinus* (DP) allergen. Sixty-three dogs (96.9%) were sensitive to at least one of the allergens in that group. Forty dogs (61.5%) were observed to have positive reactions to DF, DP and house dust. Sensitivity to house dust, in the absence of DF and/or DP was rare (3 of 65, 4.6%). In 48 cases (73.9%), the skin reactivity was limited to these 3 allergens. Seventeen dogs (26.2%), in addition, were sensitive to other allergens in varying combinations.

3.1.2. Normal dogs

Positive reactions to DF, house dust and DP allergens were observed in 22 (91.7%), 20 (83.3%) and 10 (41.7%), respectively, of the 24 normal dogs. Twenty-two dogs (91.7%) gave positive reactions to one allergen in this group. Nine dogs (37.5%) were sensitive to all three allergens. Dogs that had positive reactions to house dust were also sensitive to DF. Positive reactions restricted to these allergens were observed in 21 dogs (87.5%). A sole positive reaction to DF alone was noticed in one dog. In addition, two dogs were sensitive to flea allergen and one to yellow dock. Only two dogs (8.3%) showed no skin test reactivity to any of the allergens.

3.2. ELISA

3.2.1. Specificity of the anticanine IgE and anticanine IgGd ELISAs

IgE to DF was no longer detectable in the positive control after the serum was heated to 56°C for 4 h, whereas the detectable level of IgGd was substantially unaffected.

3.2.2. Reproducibility

The mean intra-assay CV for the IgE assay was 3.2%, whereas the mean inter-assay CV was 7.3%. For the IgGd assay, the intra-assay and inter-assay CV were 3.8 and 7.9%, respectively. The correlation coefficients of the standard curve were generally of the order of 0.995 and were always > 0.987.

3.2.3. Allergen-specific IgE

In all the cases, the RAU of the negative control serum was always below the EPSC with the exception of house dust, flea and *A. Tenuis* allergens (Table 2).

Again, with the exception of house dust, flea and *A. Tenuis* allergen-specific assay, the OD of the lowest dilutions of the standard curve were always >1.0. The mean OD in the case of house dust, flea and *A. Tenuis* were 0.3, 0.6 and 0.5, respectively.

(1) *Atopic dogs*: Detectable allergen-specific IgE was found to at least one allergen in all the cases. Allergens with the greatest frequency of positive reactions were house dust (100.0%), DF (86.2%), DP (84.6%), *A. Tenuis* (44.6%) and flea (35.4%). Allergens with a lower frequency of positive reactions were timothy grass (9.2%), common mugwort (3.1%), American beech, tag alder, yellow dock, kentucky blue and meadow fescue grass (1.5% in each case).

(2) *Normal dogs*: Similarly to the atopic dogs, positive reactions were most frequently seen with house dust (100.0%), DF (95.8%), flea (91.7%), DP (83.3%), *A. Tenuis* (33.3%) and timothy grass (16.7%). However, only one positive result, common mugwort (4.2%), was seen to any other allergen.

Table 2

Frequency of detectable allergen-specific IgE by means of ELISA expressed as number of dogs (*n*) and percentage (%), assuming a positive as $\geq$ EPSC

Allergens	Atopic dogs		Normal dogs	
	<i>n</i>	(%)	<i>n</i>	(%)
DF	56	86.2	23	95.8
DP	55	84.6	20	83.3
House dust	65	100.0	24	100.0
Flea	23	35.4	22	91.7
American beech	1	1.5	0	0.0
Tag alder	1	1.5	0	0.0
Yellow dock	1	1.5	0	0.0
Common mugwort	2	3.1	1	4.2
Kentucky blue	1	1.5	0	0.0
Meadow fescue	1	1.5	0	0.0
Timothy	6	9.2	4	16.7
<i>Alternaria tenuis</i>	29	44.6	8	33.3

3.2.4. Allergen-specific IgGd

The RAUs of the negative control serum were below the EPSC in all the cases (Table 3). It was observed in all the cases that the OD of the lowest dilutions of the standard curves were >1.0 with the exception of house dust- and flea-specific assays. The mean OD for these two assays was 0.5 and 0.6, respectively.

(1) *Atopic dogs*: Similarly to IgE, allergen-specific IgGd was detected to at least one allergen in all the cases. The highest frequencies of positive results were DF (89.2%), house dust (86.2%), DP (83.1%) and American beech (58.5%). The incidence of positive

Table 3

Frequency of detectable allergen-specific IgGd by means of ELISA expressed as number of dogs (*n*) and percentage (%) assuming a positive as $\geq$ EPSC

Allergens	Atopic dogs		Normal dogs	
	<i>n</i>	(%)	<i>n</i>	(%)
DF	58	89.2	23	95.8
DP	54	83.1	20	83.3
House dust	56	86.2	15	62.5
Flea	7	10.8	3	12.5
American beech	38	58.5	0	0.0
Tag alder	15	23.1	0	0.0
Yellow dock	10	15.4	1	4.2
Common mugwort	17	26.2	0	0.0
Kentucky blue	13	20.0	1	4.2
Meadow fescue	11	16.9	1	4.2
Timothy	15	23.1	1	4.2
<i>Alternaria tenuis</i>	19	29.2	0	0.0

reactions to other allergens, was *A. Tenuis* (29.2%) and common mugwort (26.2%), tag alder (23.1%), timothy (23.1%), kentucky blue (20.0%), meadow fescue (16.9%), yellow dock (15.4%) and flea (10.8%).

(2) *Normal dogs*: The greatest frequency of positive results in the case of allergen-specific IgGd was found in DF (95.8%), DP (83.3%), house dust (62.5%) and flea (12.5%). However, in contrast to atopic dogs, positive results with other allergens were rare. Specific IgGd to yellow dock, common mugwort, meadow fescue, and timothy grass was found only in one case each (4.2%).

3.2.5. *Correlation between serum allergen-specific IgE and IgGd*

The correlation between IgE and IgGd was examined in atopic and normal dogs in respect of the allergens in which the highest number of positive results were obtained, that is, DF and DP.

In the atopic group, allergen-specific IgGd was significantly correlated ($p < 0.01$) with the corresponding allergen-specific IgE level in the case of DF (Fig. 1(A)) and DP (Fig. 1(B)).

However, in the normal group only antibody to DF was noted to have significant correlation ($p < 0.01$) in respect of the levels of serum allergen-specific IgE (Fig. 2(A)) and IgGd (Fig. 2(B)).

3.2.6. *Comparison of IgE and IgGd levels in atopic and normal sera*

The allergen-specific antibody levels in the atopic and the normal dog sera were compared in respect to the two allergens with the highest frequency of positive reactions, that is, DF and DP.

In the case of IgE the median levels for both DF and DP were higher in the atopic dogs than in normal dogs (171.5 and 126.0 RAU for DF; 139.7 and 96.3 RAU for DP). However, these differences were not significant.

In the case of IgGd, the median levels were actually lower in the atopic dogs than in the normal dogs for both the antigens (126.7 and 247.4 RAU for DF; 58.3 and 192.5 RAU for DP). The only statistically significant difference was in respect of DP-specific IgGd levels which were significantly higher in normal dogs in comparison with atopic dogs ($p < 0.05$) (Table 4).

Table 4
Antibody levels in RAUs to DF and DP in atopic and normal dog sera

Antibody class	IgE		IgGd	
	Median (range) RAU		Median (range) RAU	
Allergen	Atopic dogs	Normal dogs	Atopic dogs	Normal dogs
DF	171.5 (31.3–7648.6)	126.0 (31.3–17787.4)	126.7 (7.8–6615.8)	247.4 (7.8–182432.0)
DP	139.7 (31.3–3695.4)	96.3 (31.3–816.7)	58.3 ^a (7.8–8559.2)	192.5 (7.8–159881.6)

^a Significantly less than normal dogs ($p < 0.05$, Mann–Whitney).

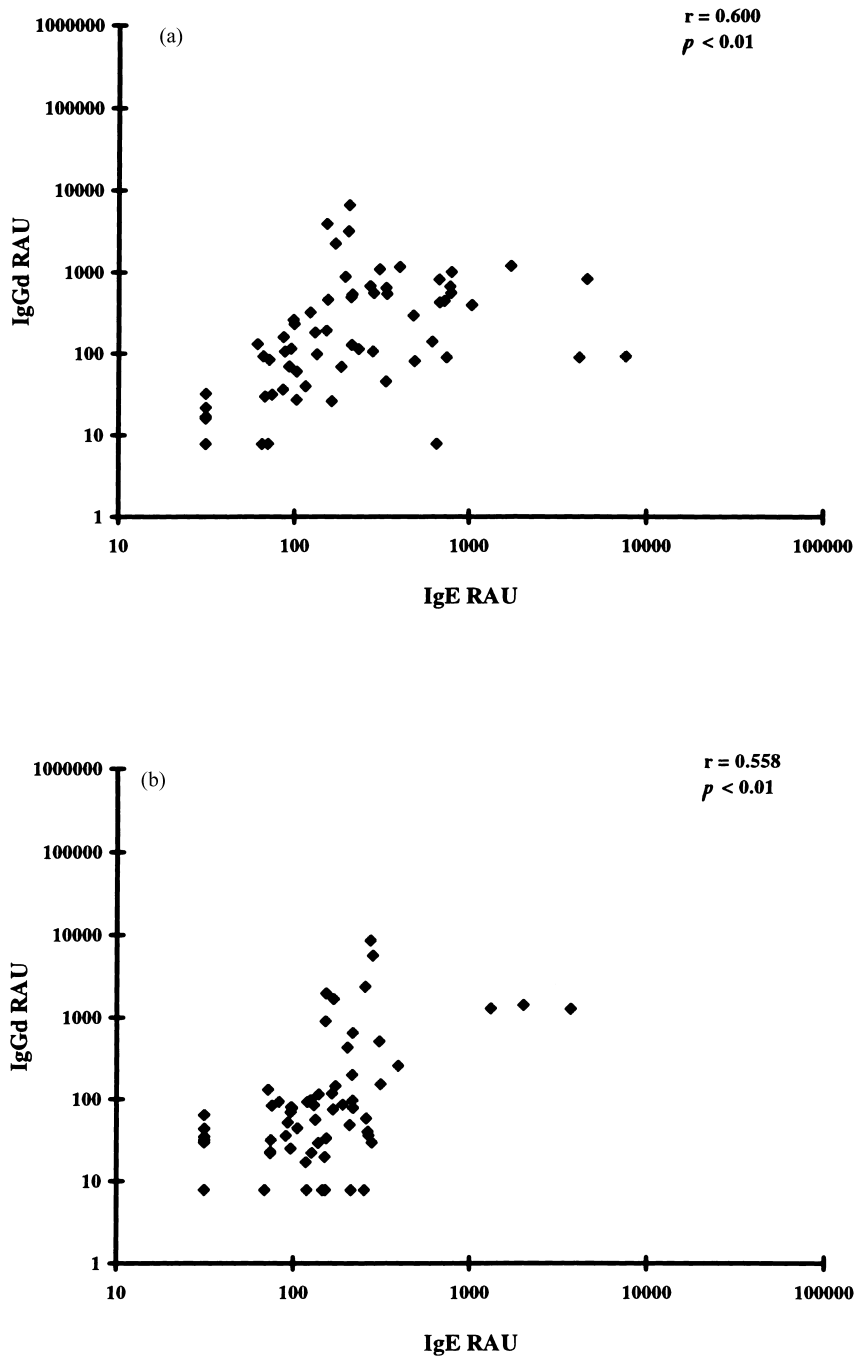


Fig. 1. Correlation between the serum allergen-specific IgE and IgGd to (a) DF and (b) DP in 65 atopic dogs. Each set of data was examined by Spearman's rank correlation coefficient.

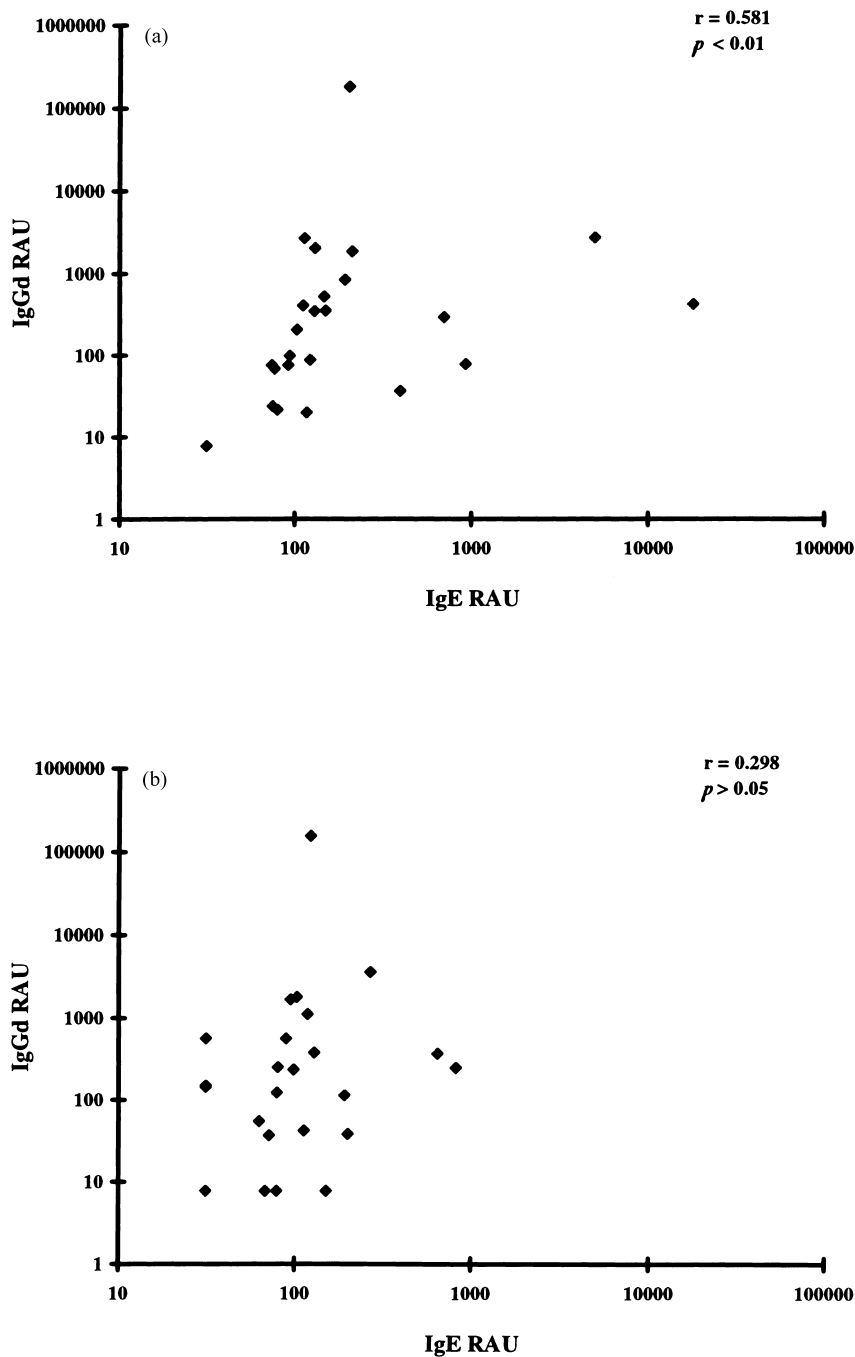


Fig. 2. Correlation between the serum allergen-specific IgE and IgGd to (a) DF and (b) DP in 24 normal dogs. Each set of data was examined by Spearman's rank correlation coefficient.

3.2.7. Correlation between the allergen-specific IgE and IgGd levels and skin test reactions

The correlation between the strength of the skin test result and the antibody level in RAU was examined for DF and DP in the atopic and normal dogs separately (Figs. 3 and 4) and in the total population (Figs. 5 and 6). In the case of DF in the total population, generally higher values of IgE and IgGd were seen in dogs which had strong positive skin tests (Fig. 5), and there was a significant difference between the level of DF-specific IgE in dogs with negative IDSTs and all those with IDST results that were graded $\geq +2$. However, in the case of IgGd, a significant difference was only observed between the level of DF-specific IgGd in dogs with negative IDSTs and those with a positive test graded +4.

In the case of DP in the total population (Fig. 6), again, with the exception of +1 IDST results, a significant difference between the level of IgE in dogs with negative IDSTs and all other grades of positive reactions was seen. However, there was no significant difference between the levels of IgGd in dogs with negative IDSTs and any grade of positive reaction.

3.2.8. Specificity, sensitivity and positive and negative predictive values

Using the cut-off point as the EPSC, the sensitivity with respect to DF was 92.5%, respectively, for IgE, and 92.5% for IgGd (Table 5). Similarly, the specificity was 44.4%, but lower at 22.2% for IgGd.

Table 5
Sensitivity, specificity and positive and negative predictive values of ELISA

	Number IDST + ve	Sensitivity (%)		Specificity (%)		PPV (%)		NPV (%)	
		IgE	IgGd	IgE	IgGd	IgE	IgGd	IgE	IgGd
<i>Mites/insects</i>									
DF	80	92.5	92.5	44.4	22.2	93.7	91.4	40.0	25.0
DP	55	92.7	85.5	29.4	20.6	68.0	63.5	71.4	46.7
House dust	76	100.0	80.3	0.0	23.1	85.4	85.9	0.0	16.7
Flea	8	50.0	12.5	49.4	88.9	8.9	10.0	90.9	91.1
<i>Trees</i>									
Tag alder	3	33.3	33.3	100.0	83.7	100.0	6.7	97.7	97.3
American beech	3	33.3	66.7	100.0	58.1	100.0	5.3	97.7	98.0
<i>Weeds</i>									
Yellow dock	7	14.3	57.1	100.0	91.5	100.0	36.4	93.2	96.2
Common mugwort	7	0.0	42.9	96.3	82.9	0.0	17.6	91.9	94.4
<i>Grasses</i>									
Kentucky	5	20.0	40.0	100.0	85.7	100.0	14.3	95.5	96.0
Meadow fescue	8	12.5	37.5	100.0	88.9	100.0	25.0	92.0	93.5
Timothy	3	0.0	66.7	88.4	83.7	0.0	12.5	96.2	98.6
<i>Mould</i>									
<i>Alternaria tenuis</i>	1	100.0	100.0	59.1	79.5	2.7	5.3	100.0	100.0

Data were assessed by assuming a positive result as the end point of the standard curve (EPSC).

PPV, Positive predictive value.

NPV, Negative predictive value.

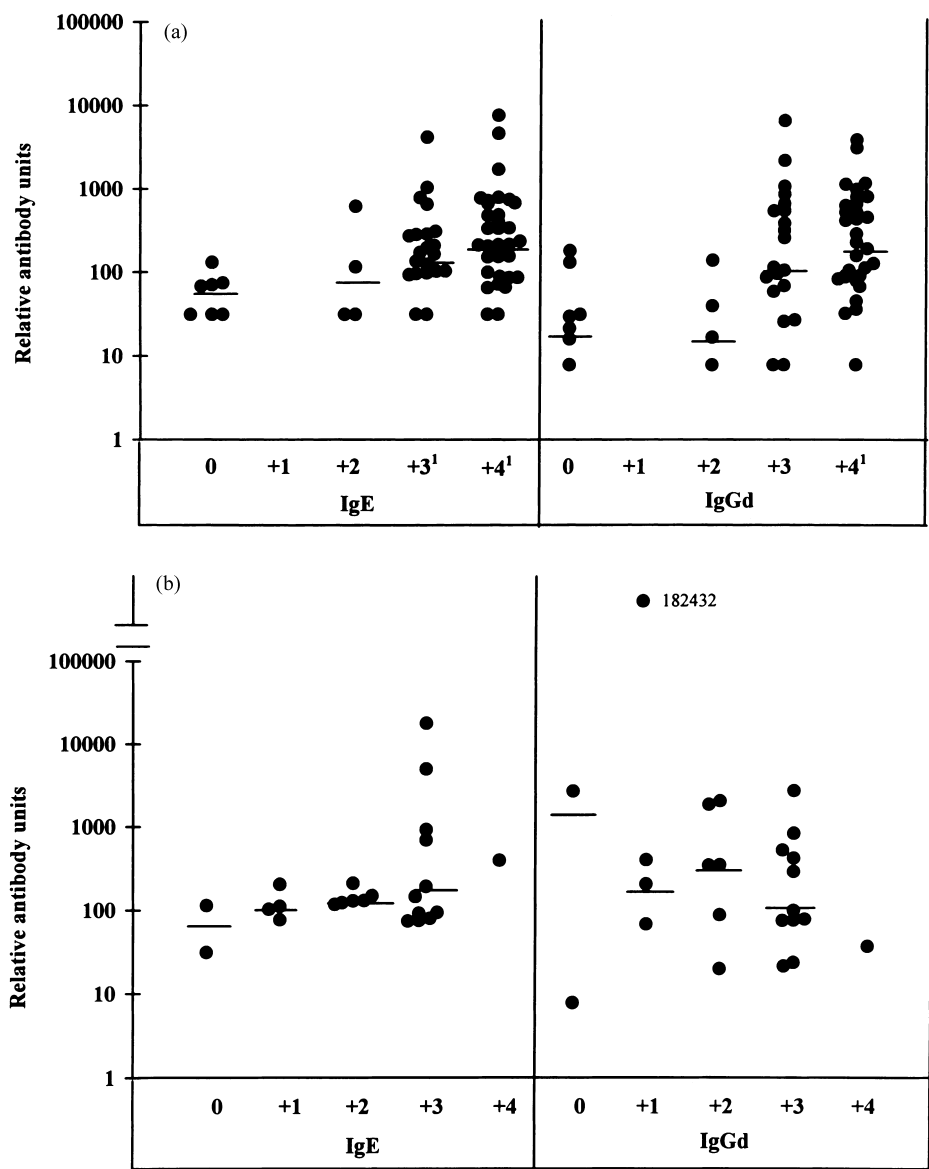


Fig. 3. Correlation between the results of skin tests and the levels of IgE and IgGd to DF in (a) 65 atopic dogs and (b) 24 normal dogs. Bar represents the median. ¹ Significantly higher than the level of DF-specific antibodies in dogs with negative skin (0), ($p < 0.05$, Kruskal–Wallis one-way ANOVA).

Similar results were obtained for DP. The sensitivity for IgE was 92.7% and for IgGd, was 85.5%. Specificity was 29.4% for IgE and again lower at 20.6% for IgGd. The positive and negative predictive values varied with these two allergens, but were not dramatically different.

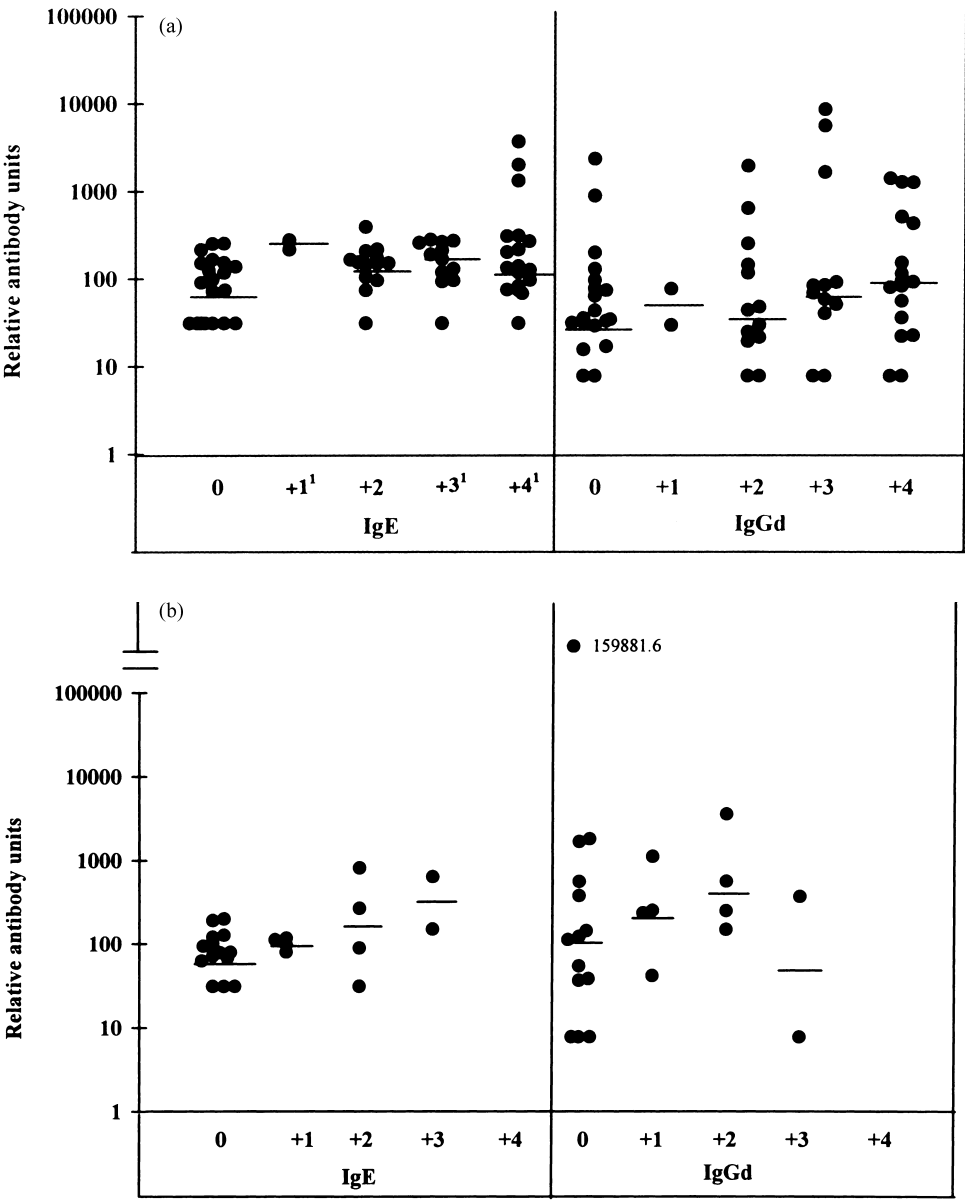


Fig. 4. Correlation between the results of skin tests and the levels of IgE and IgGd to DP in (a) 65 atopic dogs, and (b) 24 normal dogs. Bar represents the median. ¹ Significantly higher than the level of DP-specific antibodies in dogs with negative skin tests (0), ($p < 0.05$, Kruskal–Wallis one-way ANOVA).

In the case of house dust and flea antigens, disappointing results were noted with sensitivities ranging from 50–100% for IgE and 12–80% for IgGd. Specificities likewise showed marked variation from 0–59% for IgE and 23–88% for IgGd.

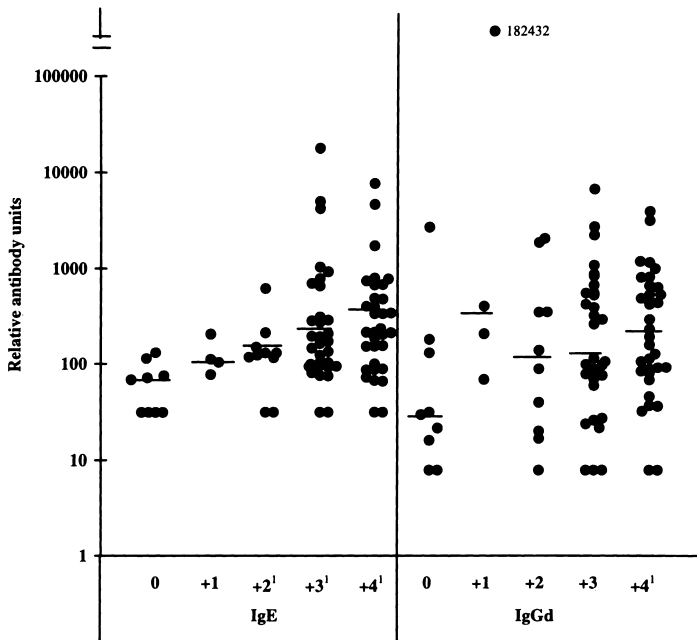


Fig. 5. Correlation between the results of skin tests and the levels of IgE and IgGd to DF in the total population ($n = 89$). Bar represents the median. ¹ Significantly higher than the level of DF-specific antibodies in dogs with negative skin tests (0), ($p < 0.05$, Kruskal–Wallis one-way ANOVA).

Similar analyses were undertaken for pollen antigens. In general, specificity and negative predictive values were high and of the order of 80–100% for all allergens and both the isotypes.

Sensitivity was generally higher for IgGd, but the positive predictive values were very much higher for IgE in five out of the seven pollens and slightly higher for IgGd in the case of two.

The very low number of positive IDST reactions to *A. Tenuis* ($n = 1$) precluded meaningful analyses.

4. Discussion

The results of the IDSTs in atopic dogs in this study were consistent with a previous publication (Sture et al., 1995). House dust and house dust mites appear to be the most important allergens in atopic dogs in the UK. Since house dust is a complex allergen which contains several dust mite residues, it has been suggested that house dust mites, particularly *Dermatophagoides* species, could be the major allergens in house dust (Wellington et al., 1991b; Sture et al., 1995). The rare incidence of sensitivity to house dust in the absence of reactions to house dust mites in this study supports their findings.

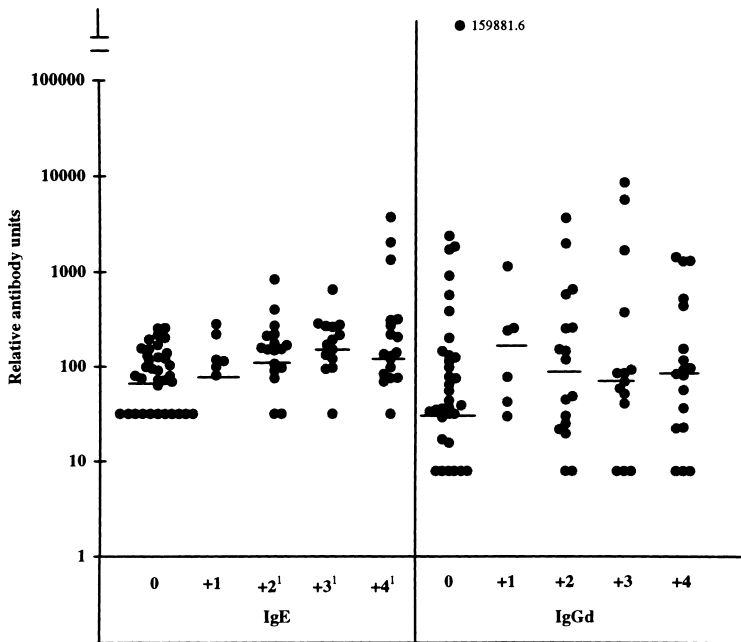


Fig. 6. Correlation between the results of skin tests and the levels of IgE and IgGd to DP in the total population ($n=89$). Bar represents the median. ¹ Significantly higher than the level of DP-specific antibodies in dogs with negative skin tests (0), ($p < 0.05$, Kruskal–Wallis one-way ANOVA).

Studies determining the optimal skin threshold concentrations of house dust and house dust mite extracts showed conflicting results. August (1982) found that the concentration recommended by Willemse and van den Brom (1982), that is, 250 PNU ml⁻¹, gave false-positive reactions in 54 of the 90 clinically normal dogs. On the other hand, Scott (1981) reported that only eight of the 100 suspected atopic dogs had obvious false-positive reactions at a concentration of 1000 PNU ml⁻¹. Wellington et al. (1991a) showed that a concentration of 31.25 PNU ml⁻¹ was found to be the maximum, non-irritant strength for house dust mite allergens in normal dogs. Interestingly, in the latter study, 11 of the 34 research animals failed to show positive reactions to any of the six tested dilutions of the house dust mite allergen. In contrast, only one privately owned dog of 22 failed to give a positive reaction. This could be interpreted as reflecting differences in antigen exposure.

Codner and Tinker (1995) reported that 14 of the 24 dogs (58%) and 12 of the 24 dogs (50%) without allergic skin disease showed positive intradermal test reactivity to house dust mite (a mixed DP and DF allergen), and house dust allergen, respectively, when used at the manufacturer's recommended testing concentration; that is, 1:5000 w/v for house dust mite and 100 PNU ml⁻¹ for house dust. In contrast, Curtis et al. (1996) found a very low incidence of positive skin test reactivity to DF and DP in 10 healthy kennelled dogs which were presumably house dust mite-naïve, with only one positive reaction to each allergen. The allergen extracts were obtained from the same manufacturer as in the study above, and were used at 1 : 2000 w/v.

The reasons for inconsistency in the above findings could be multifaceted. The composition of allergen extracts from the different manufacturers can vary considerably in their allergen content, depending on the source of the material, the extraction procedure and the storage conditions (Reedy and Miller, 1989). A uniform method of standardisation that accurately measures the biological potency does not exist. Therefore, it is difficult to compare the claims of statements regarding the optimal skin testing threshold concentration of house dust and mite allergens from one study to another. However, the higher number of positive reactions to these allergens in privately owned healthy dogs than in research animals could indicate clinically irrelevant responses rather than irritant 'false-positive' results.

The findings of a surprisingly high incidence of positive reactions to house dust mites, particularly DF, in the normal dog population in this study will inevitably cast doubt upon the concentration employed (1 : 10 000 w/v for DF and DP; 1000 PNU for house dust). However, this is the first study in which clinically normal dogs were both skin-tested and blood-sampled for IgE ELISA, and the high frequency of detectable allergen-specific IgE in the subsequent ELISA supports the specificity of the IDST reactions.

The low mean value of intra-assay and inter-assay CV for this study and the reproducibility of highly sensitive standard curves in all assays (with the exception of flea and house dust in the IgGd assays and flea, house dust and *A. Tenuis* in the IgE assays) and the consistency of the correlation coefficients obtained support the precision and reliability of quality control in the ELISAs in this study.

Although the reproducibility was still good in the case of assays for house dust, flea and *A. Tenuis*, the results for these allergens were disappointing when the specificity, sensitivity and positive and negative predictive values were examined. At the lowest dilutions of the standard curve the OD values were <0.6, in the case of the allergen-specific IgE and IgGd assays to house dust and flea allergen, which resulted in a relatively flat and insensitive standard curve. Furthermore, the reliability of these assays was less favourable as the OD of the negative control serum was consistently above the EPSC which implies a failure to discriminate between the negative control serum and the positive standard serum. All these tend to suggest a heterogeneity and complexity of the above allergens, and are consistent with the widely held view that only a minor proportion of these complex mixtures consists of specific allergen. Their use in *in vitro* tests can, thus, not be recommended without further purification.

In contrast to *A. Tenuis*-specific IgE assays that gave the same poor results as did the flea and house dust assays, *A. Tenuis*-specific IgGd produced a steep standard curve similar to that obtained in mite- and pollen-specific assays. This could be explained by the possibility that IgE and IgGd antibodies recognised differing antigens in the mixtures or different epitopes. Further investigations using western blots could refute or substantiate this possibility.

The high frequency of detectable IgE-specific antibody to DF and DP in the atopic population, again stresses the important role of these mites in canine atopic disease.

Similarly, a high frequency of DF- and DP-specific IgE was also detected in the normal dog population. These findings correlated well with the *in vivo* IDST results. There are a number of previous reports describing positive allergen-specific IgE ELISA results in

normal dogs, but all of them showed a sharp disagreement with IDST results using commercially available kits (Codner and Lessard, 1993; Bond et al., 1994; Day et al., 1996). However, only one of these studies compared results of IgE levels and IDSTs in both the atopic and normal populations. The majority of the studies stresses the lack of specificity of in vitro tests, that is, their propensity to give 'false-positive' reactions in normal dogs, particularly to mite allergens.

However, a closer examination of this issue revealed that there were technical and design flaws in some of the studies. The results can be explained, in part, by differences between the allergen extracts used in in vivo and in vitro assays (Codner and Lessard, 1993; Paradis and Lecuyer, 1993). Indeed one study showed that there are differences in terms of sensitivity and specificity of products from different manufacturers (Bond et al., 1994). In addition, such a flaw could occur if the test reagents are not completely IgE-specific, that is, they detect IgG or other non-IgE antibodies.

Also, some of the studies, unfortunately, did not perform IDSTs on the normal dog (control) population, which meant that the claims of false-positive serologic findings may be inappropriate (Bond et al., 1994; Day et al., 1996).

The high levels of IgE to DF and DP in all populations could, at least in part, result from the protease activity of mites that could disrupt the IgE network as suggested by Hewitt et al. (1995). These authors showed that the protease activity of DP group I (*Der p* I) was able to cleave the low-affinity IgE receptor (CD23) from the surface of human B lymphocytes, selectively. It was suggested that the loss of cell surface CD23 from IgE-secreting cells could promote and enhance IgE immune responses by ablating an important feedback inhibitory mechanism that normally limits IgE synthesis. Furthermore, since soluble CD23 is reported to promote the IgE production (Sutton and Gould, 1993), fragments of CD23 released by *Der p* I would directly enhance the synthesis of IgE.

The frequency of both positive IDST and IgE ELISAs to mite allergens observed in the normal dog population in this study leads one to question the value of either test in the diagnosis of atopic disease. It may also cast doubt upon the role of IgE in the pathogenesis of the disease.

The most rational explanation for the latter question would be the existence of heterogeneity of IgE in dogs. MacDonald et al. (1987) proposed that heterogeneity of IgE may exist in man, in which two basic types of IgE would exist: IgE+ and IgE-. IgE+ was defined as molecules that are capable of interacting with certain co-factors, termed histamine releasing factors (HRF), to induce activation of basophils. IgE- molecules are fully capable of binding to basophils, but cannot as readily induce cellular activation. Development of a severe allergic disease would correlate with the presence of IgE+. The recent identification and cloning of a specific HRF that is synergistic with IgE from IgE+ (but not IgE-) donors is evidence supporting this hypothesis (MacDonald et al., 1995).

Two recent studies in dogs provided evidence that IgE subclasses may also exist in this species. Peng et al. (1997) demonstrated the existence of two populations of canine IgE which differed from each other by charge and affinity on the basis of the standard protein separation techniques. However, both of them were capable of inducing 48 h PK tests. Jackson et al. (1996) studied the leukocyte histamine release in atopic dogs, normal dogs and artificially sensitised dogs and found that histamine release to antigen or to anti-IgE

was generally found only in atopic dogs, irrespective of the amount of total or allergen-specific IgE.

The other important question raised by these studies is, how reliable are either IDSTs or *in vitro* assays in the diagnosis of atopic disease. Most clinicians claim that evidence of allergen-specific IgE, assessed either by ELISA or by IDST, is merely confirmatory and should not be used to make the diagnosis. However, so long as immunotherapy is still a preferred treatment, identification of the allergens to which the patient is sensitive is clearly of value.

In determining whether assays of allergen-specific IgE or IgGd are more reliable in terms of correlating with the IDST, a close perusal of the sensitivity, specificity and positive and negative predictive values is necessary. In atopic dogs, IgGd antibody was frequently detected to pollen allergens in the absence of positive IDSTs or positive IgE ELISA results which led to a low-positive predictive value in the case of IgGd to these allergens. This could be similar to the abnormal IgG4 antibody response to aeroallergens in atopic patients as has been observed in two studies in man (Kemedly et al., 1989; Jeannin et al., 1994). However, the underlying cause is unclear and it is equally unclear whether they have any pathogenic significance.

It was also noteworthy that the levels of IgGd to DF and DP were actually higher in normals than in atopics, although the difference was significant only in the case of DP. These findings are in accordance with the previous studies on man, where allergen-specific IgG4 concentrations in non-allergic subjects are always significantly higher than in allergic patients (Homburger et al., 1986; Lee et al., 1988). This could imply that IgGd in dogs is an indicator of chronic antigenic stimulation to environmental allergens to which the individual is continually exposed in high concentrations.

However, it was also noteworthy that the IgE and IgGd antibody levels to both DF and DP in atopic dog sera were positively correlated.

5. Conclusions

In conclusion, the present study showed that there is a high incidence of positive IDSTs to house dust and mite allergens in normal dogs. These positive skin tests are usually accompanied by positive IgE ELISA results and, therefore, do not represent false positive reactions. The findings of positive IDSTs and IgE ELISAs in normal dogs are consistent with the possible heterogeneity of canine IgE, with a subtype being associated with allergic disease. The levels of IgE and IgGd were correlated in the case of DF and DP in atopic dogs and in the case of DF only in normal dogs. There was a consistent tendency for dogs with stronger skin tests to give higher IgE levels. The specificity, sensitivity and positive and negative predictive values of IgE and IgGd were similar for the mite allergens. However, positive IgGd results were frequently found in pollen antigens leading to a generally low positive predictive value in the case of those allergens. These results are not supportive of a role for IgGd in the pathogenesis of atopic disease in dogs. Indeed, the significantly higher levels of DP-specific IgGd levels in normal dogs as compared with atopic dogs, would argue for a protective role for this immunoglobulin subclass.

Acknowledgements

The financial support of the Waltham Centre for Pet Nutrition is gratefully acknowledged. The authors would like to thank Mr R.G. Harvey, of Godiva Referrals, Coventry, and Dr K.L. Thoday, Dr P.B. Hill, Mr T. Nuttall and Mr A.H.M. van den Broek, of the University of Edinburgh, Royal (Dick) School of Veterinary Studies, for supplying the sera from atopic dogs.

References

- Aalberse, R.C., van Milligen, F., Tan, K.Y., Stapel, S.O., 1993. Allergen-specific IgG4 in atopic disease. *Allergy* 48, 559–569.
- August, J.R., 1982. The reaction of canine skin to the intradermal injection of the allergenic extracts. *J. Am. Anim. Hosp. Assoc.* 19, 157–163.
- Bond, R., Thorogood, S.C., Lloyd, D.H., 1994. Evaluation of two enzyme-linked immunosorbent assays for the diagnosis of canine atopy. *Vet. Rec.* 135, 130–133.
- Codner, E.C., Lessard, P., 1993. Comparison of intradermal allergy test and enzyme-linked immunoassay in dogs with allergic skin disease. *J. Am. Vet. Med. Assoc.* 202, 739–743.
- Codner, E.C., Tinker, M.K., 1995. Reactivity to intradermal injections of extracts of house dust and house dust mite in healthy dogs and dogs suspected of being atopic. *J. Am. Vet. Med. Assoc.* 206, 812–816.
- Curtis, C.F., Bond, R., Lloyd, D.H., 1996. Evaluation of the response to intradermally injected environmental mite solutions in healthy kennelled dogs, In: *Proc. Third World Congress of Veterinary Dermatology*, Edinburgh, UK, p. 52.
- Day, M.J., Corato, A., Shaw, S.E., 1996. Subclass profile of allergen-specific IgG antibodies in atopic dogs. *Res. Vet. Sci.* 61, 136–142.
- Desvaux, F.X., Peltre, G., David, B., 1989. Characteristic of grass pollen-specific IgE, IgA, IgM classes and IgG subclasses in allergic patients. *Int. Arch. Allergy Appl. Immunol.* 89, 281–287.
- Engvall, E., Perlmann, P., 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* 109, 129–135.
- Gerstman, B.B., Cappucci, D.T., 1986. Evaluating the reliability of diagnostic test results. *J. Am. Vet. Med. Assoc.* 188, 248–251.
- Halliwell, R.E.W., Schwartzman, R.M., Rockey, J.H., 1972. Antigenic relationship between human IgE and canine IgE. *Clin. Exp. Immunol.* 10, 399–407.
- Halliwell, R.E.W., Longino, S.J., 1985. IgE and IgG antibodies to flea antigen in differing dog populations. *Vet. Immunol. Immunopath.* 8, 215–223.
- Halliwell, R.E.W., Gorman, N.T., 1989. Atopic diseases, In: Halliwell, R.E.W., Gorman, N.T., (Eds), *Veterinary Clinical Immunology*, W.B. Saunders, Philadelphia, pp. 232–252.
- Halliwell, R.E.W., 1990. Clinical and immunological aspects of allergic skin diseases in domestic animals, In: von Tscherner, C., Halliwell, R.E.W., (Eds.), *Advances in Veterinary Dermatology*, vol. 1, Balliere Tindall, London, pp. 91–117.
- Halliwell, R.E.W., McGorum, B.C., Irving, P., Dixon, P.M., 1993. Local and systemic antibody production in horses affected with chronic obstructive pulmonary disease (COPD). *Vet. Immunol. Immunopath.* 38, 201–215.
- Hewitt, C.R.A., Brown, A.P., Hart, B.J., Pritchard, D.I., 1995. A major house dust mite allergen disrupts the immunoglobulin E network by selectively cleaving CD23: Innate protection by antiproteases. *J. Exp. Med.* 182, 1537–1544.
- Hill, P.B., Moriello, K.A., DeBoer, D.J., 1995. Concentrations of total serum IgE, IgA, and IgG in atopic and parasitized dogs. *Vet. Immunol. Immunopath.* 44, 105–113.
- Hites, M.J., Kleinbeck, M.L., Loker, J.L., Lee, K.W., 1989. Effect of immunotherapy on the serum concentration of allergen-specific IgG antibodies in dog sera. *Vet. Immunol. Immunopath.* 22, 39–51.

- Homburger, H.A., Mauer, K., Sachs, M.I., O'Connell, E.J., Jacob, G.L., Caron, J., 1986. Serum IgG4 concentrations and allergen-specific IgG4 antibodies compared in adults and children with asthma and nonallergic subjects. *J. Allergy Clin. Immunol.* 77, 427–434.
- Jackson, H.A., Miller, H.R.P., Halliwell, R.E.W., 1996. Canine leukocyte histamine release: Response to antigen and to anti-IgE. *Vet. Immunol. Immunopath.* 53, 195–206.
- Jeannin, P., Delneste, Y., Tillie-Leblond, I., Wallaert, B., Carlier, A., Pestel, J., Tonnel, A.B., 1994. Abnormal IgG4 antibody response to aeroallergens in allergic patients. *Int. Arch. Allergy Appl. Immunol.* 104, 191–198.
- Kemedy, D.M., Urbanek, R., Ewan, P., McHugh, S., Richards, D., Patel, S., Lessof, H.M., 1989. The subclass of IgG antibody in allergic disease. II: The IgG subclass of antibodies produced following natural exposure to dust mite and grass pollen in atopic and non-atopic individuals. *Clin. Exp. Allergy* 19, 545–549.
- Lee, D., Edwards, R.G., Dewdney, J.M., 1988. Measurement of IgG subclasses in the sera of laboratory workers exposed to rats. *Int. Arch. Allergy Appl. Immunol.* 87, 222–224.
- Lucas, A.H., 1990. IgG subclass-restricted immune responses to allergens.. *Springer Semin. Immunopath.* 12, 385–400.
- MacDonald, S.M., Lichtenstein, L.M., Proud, D., Plaut, M., Naclerio, R.M., MacGlashan, D.W., Kagey-Sobotka, A., 1987. Studies of IgE-dependent histamine releasing factors: Heterogeneity of IgE. *J. Immunol.* 139, 506–512.
- MacDonald, S.M., Rafnar, T., Langdon, J., Lichtenstein, L.M., 1995. Molecular identification of an IgE-dependent histamine releasing factor. *Science* 269, 688–690.
- Mazza, G., Duffus, W.P.H., Elson, C.J., Stokes, C.R., Wilson, A.D., Whiting, A.H., 1993. The separation and identification by monoclonal antibodies of dog IgG fractions. *J. Immunol. Methods* 161, 193–203.
- Mazza, G., Whiting, A.H., Day, M.J., Duffus, W.P.H., 1994. Preparation of monoclonal antibodies specific for the subclasses of canine IgG. *Res. Vet. Sci.* 57, 140–145.
- Paradis, M., Lecuyer, M., 1993. Evaluation of an in-office allergy sceening test in nonatopic dogs having various intestinal parasites. *Can. Vet. J.* 34, 293–295.
- Peng, Z., Arthur, G., Rector, E.S., Kierck-Jaszczuk, D., Simons, F.E.R., Becker, A.B., 1997. Heterogeneity of polyclonal dog IgE characterized by differential charge, affinity to protein A, and antigenicity. *J. Allergy Clin. Immunol.* 100, 87–95.
- Reedy, L.M. and Miller, W.H., 1989. *Allergic Skin Disease of Dogs and Cats*, W.B. Saunders, Philadelphia, PA.
- Schwartzman, R.M., 1984. Cutaneous amyloidosis associated with monoclonal gammopathy. *J. Am. Vet. Med. Assoc.* 185, 102–104.
- Scott, D.W., 1981. Observations on canine atopy. *J. Am. Anim. Hosp. Assoc.* 17, 91–100.
- Shakib, F., McLaughlan, P., Stanworth, D.R., Smith, E., Fairburn, E., 1977. Elevated serum IgE and IgG4 in patients with atopic dermatitis. *Br. J. Dermatol.* 97, 59–63.
- Sture, G.H., Halliwell, R.E.W., Thoday, K.L., van den Broek, A.H.M., Henfry, J.I., Lloyd, D.H., Mason, I.S., Ferguson, E., 1995. Canine atopic disease: The prevalence of positive intradermal skin tests at two sites in the north and south of Great Britain. *Vet. Immunol. Immunopath.* 44, 293–308.
- Sutton, B.J., Gould, H.J., 1993. The human IgE network. *Nature* 366, 421–428.
- Wellington, J.R., Miller, W.H., Scarlett, J.M., 1991a. Dertermination of skin threshold concentration of an aqueous house dust mite allergen in normal dogs. *Cornell Vet.*, 81, 37–42.
- Wellington, J.R., Miller, W.H., Erb, H.N., 1991b. Dermatophagoides mites in house dust as an allergen source in atopic dogs. *Cornell Vet.*, 81, 429–434.
- Wheeler, A.W., 1993. Grass pollen specific antibody in the plasma of normal dogs. *Lab. Anim.* 27, 73–76.
- Willemse, A., van den Brom, W.E., 1982. Evaluation of the intradermal allergy in normal dogs. *Res. Vet. Sci.* 32, 57–61.
- Willemse, A., Noordzij, A., Rutten, V.P.M.G., Bernadina, W.E., 1985a. Induction of non-IgE anaphylactic antibodies in dogs. *Clin. Exp. Immunol.* 59, 351–358.
- Willemse, A., Noordzij, A., Van den Brom, W.E., Rutten, V.P.M.G., 1985b. Allergen specific IgGd antibodies in dogs with atopic dermatitis as determined by the enzyme-linked immunosorbent assay (ELISA). *Clin. Exp. Immunol.* 59, 359–363.