

Short communication

Peripheral blood mononuclear cell responses to *Dermatophagoides farinae* in canine atopic dermatitis

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Abstract

Atopic dermatitis is a chronic inflammatory and pruritic skin disease commonly seen in dogs and humans that is characterised by the presence of allergen-specific IgE. Data from skin tests and serological analysis suggest that the house dust mite *Dermatophagoides farinae* is the most important allergen in dogs with atopic dermatitis. The aim of this study was to determine if *D. farinae* specific peripheral blood mononuclear cell (PBMC) responses could be detected in dogs with atopic dermatitis. PBMCs were isolated by the density centrifugation from dogs with atopic dermatitis that were skin test positive for *D. farinae*, dogs with atopic dermatitis that were skin test negative for *D. farinae*, and healthy dogs. Cells were cultured with increasing concentrations of the *D. farinae* extract, no antigen, vaccine antigens or concanavalin A (ConA). There was significantly greater responsiveness of PBMCs from the *D. farinae* positive dogs than from either the *D. farinae* negative or healthy dogs (ANOVA, $P < 0.05$). In contrast, no significant differences were observed in the control responses between the three groups. This is the first study to demonstrate that *D. farinae* specific circulating memory cells are involved in the pathogenesis of canine house dust mite hypersensitivity. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Dog; Atopic dermatitis; PBMC proliferation; *Dermatophagoides farinae*

1. Introduction

Atopic dermatitis is a well-recognised chronic inflammatory skin disease of humans (Rothe and Grant-Kels, 1996) and dogs (Scott et al., 1995). The relative risk of atopic

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dermatitis is strongly correlated to the house dust mite exposure (Cooper, 1994). Between 40 and 80% of human patients have house dust mite specific serum IgE and positive skin tests (Halbert et al., 1995), and approximately 60% of atopic dogs have positive skin tests to the house dust mite allergens (Sture et al., 1995). The major house dust mite species in temperate areas are *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*. *D. pteronyssinus* prefers more humid conditions than *D. farinae* and is the most common species in the UK (Thomas et al., 1998). Despite this, skin test reactions to *D. farinae* are more frequent than to *D. pteronyssinus* in atopic dogs in the UK (Sture et al., 1995).

Canine atopic dermatitis has been viewed as an IgE/mast cell-mediated disease (Scott et al., 1995). However, IgE production is dependent on the allergen-specific CD4⁺ T_H-cells (Lydyard and Grossi, 1998) and infiltration of CD4⁺ and CD8⁺ lymphocytes is observed in lesional atopic skin in both dogs (Olivry et al., 1997) and humans (Leung, 2000). Several studies in humans have demonstrated PBMC proliferation to the crude *Dermatophagoides* extracts and purified allergens (reviewed in Leung, 2000). Despite this, investigation of PBMC responses in atopic dogs has been limited. One unpublished report suggested that PBMCs from atopic dogs had a proliferative response to the crude *D. pteronyssinus* extract and major allergens identified on Western blots (Shaw, 2000), but responses to *D. farinae* were not investigated.

The aim of this study, therefore, was to determine if allergen-specific T-cells were involved in the pathogenesis of canine atopic dermatitis by investigating PBMC responses to *D. farinae* in dogs with atopic dermatitis that were skin test positive to *D. farinae*, skin test negative to *D. farinae* and healthy dogs.

2. Materials and methods

2.1. Study population

Dogs with atopic dermatitis 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 causes of pruritus (Willemse, 1986; Nuttall et al., 1998). Coat brushings, skin scrapings and trial therapy were used to eliminate the possibility of ectoparasite infestation. All dogs underwent a 6 weeks, home cooked diet trial to eliminate the food intolerance. Seborrhoea, staphylococcal pyoderma and *Malassezia* infections were managed appropriately. No anti-inflammatory medication was given for at least 3 weeks prior to examination.

All dogs with a clinical diagnosis of atopic dermatitis were intradermally skin tested with 57 allergens, including a *D. farinae* extract (Greer Laboratories, Lenoir, USA) as follows. Dogs were sedated with 0.15 mg/kg xylazine i.m. Hair was clipped from the lateral flank and 0.05 ml of each allergen extract, 1/100,000 (w/v) histamine (positive control) and saline with phenol diluent (negative control) were injected i.d. Test sites were assessed according to standard criteria after 20 min and subjectively scored from 0 to +4 compared to the controls. Reactions greater than or equal to +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.

2.2. *D. farinae* extract

A freeze-dried crude *D. farinae* extract (Greer Laboratories, Lenoir, USA) was reconstituted in sterile PBS at 1 mg/ml (BCA protein assay; Pierce Laboratories, Rockford, USA).

2.3. Sample collection

Blood samples were collected into 1.2% tri-sodium citrate by jugular venepuncture from atopic dogs with either positive ($n = 10$) or negative ($n = 7$) intradermal skin test reactions to *D. farinae*. Blood samples from the healthy dogs ($n = 10$) were collected by cardiac puncture immediately after euthanasia. Samples were kept on ice and processed within 2 h.

2.4. PBMC separation

Blood samples were diluted 1:1 in chilled PBS/1.2% tri-sodium citrate (citrated PBS). A volume of 20 ml of each sample was carefully layered over 20 ml of 1.007 g/ml polysucrose/sodium diatrizoate solution (Histopaque[®]-1077; Sigma, Poole, UK) and centrifuged at $440 \times g$ for 20 min at 20°C. Cells were collected from the interface, washed in a $10 \times$ volume of chilled citrated PBS and centrifuged at $250 \times g$ for 10 min at 4°C. The cell pellet was re-suspended in 1 ml of sterile distilled water for 30 s before adding 9 ml of $10 \times$ PBS. The cell suspension was then washed in a $3 \times$ volume of chilled citrated PBS and centrifuged at $200 \times g$ for 10 min at 4°C. The cell pellet was re-suspended in 5 ml of Dulbecco's modified Eagle's medium (Sigma), supplemented with 10% FCS (Serotec, Kidlington, UK), 2 mM L-glutamine (Life Technologies, Paisley, UK), 50 mM 2-mercapto-ethanol (Life Technologies, Paisley, UK), 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin B ($100 \times$ antibiotic-antimycotic solution; Sigma) and 1% non-essential amino acids (Life Technologies, Paisley, UK) (hereafter, referred to as complete medium). A volume of 100 microlitres of the cell suspension was diluted 1:1 with trypan blue (Sigma, Poole, UK). Viable cells were counted in a modified Neubauer haemocytometer and the suspension adjusted to 1×10^6 cells/ml in complete medium.

2.5. PBMC cultures

The *D. farinae* extract was diluted 10-fold from 1000–0.01 micrograms/ml in complete medium. A volume of 100 microlitres of each dilution was added to the appropriate wells of a 96-well U-bottomed tissue culture plate (Nunclon Microwell; Fisher Scientific, Loughborough, UK). Wells with 100 microlitres of 1 mg/ml concanavalin A (ConA) (Sigma), 1/10 *Bordetella bronchiseptica* (Intrac[®]; Schering-Plough Animal Health, Uxbridge, UK) and 1/10,000 canine parvovirus vaccine antigens (Parvo-C[®]; Intervet UK Ltd.; Milton Keynes, UK) diluted in complete medium were included as positive controls. Wells with

100 microlitres of complete medium were included as negative control resting wells. A volume of 100 microlitres of the cell suspension (i.e. 1×10^5 cells) was added to each well. Wells with 200 microlitres of complete medium only were included as background. All wells were plated in triplicate.

Plates were cultured at 38°C in 5% CO₂ for 7 days. Methyl-³H thymidine (0.5 µCi) (Amersham-Pharmacia Biotech, Little Chalfont, UK) was added to each well for the final 4 h of culture. Optimum dilutions and culture conditions were established prior to this study (data not shown).

Cells were harvested onto a glass-fibre sheet (Harvester 96; Tomtec, Orange, USA), air dried, sealed in counting bags with 4 ml scintillant (Optiscint HiSafe; Wallac, Turku, Finland) and the activity of each well recorded as counts per min (cpm) using a beta-counter (Microbeta Plus; Wallac).

2.6. Data analysis

Results were expressed as the mean cpm per triplicate wells. Mean background cpm (wells with medium only) was subtracted from all other wells. Cell proliferation was expressed as, mean cpm (test wells-background – resting wells-background). After ensuring data were normally distributed, analyses of variance with Tukey's post tests were used to compare cell proliferation between groups (Instat; Graphpad Inc., San Diego, USA). Significance was set at $P < 0.05$.

3. Results and discussion

To our knowledge, this study documents for the first time the presence of circulating *Dermatophagoides farinae* specific T-cells in canine atopic dermatitis. PBMCs from *D. farinae* positive atopic dogs showed greater proliferation to all concentrations of the *D. farinae* extract than PBMCs from either *D. farinae* negative atopic dogs or healthy dogs (Fig. 1). Optimum proliferation was seen with 50 micrograms/ml *D. farinae*. At this concentration, there was significantly greater proliferation of PBMCs from *D. farinae* positive dogs with atopic dermatitis than either *D. farinae* negative dogs with atopic dermatitis or healthy dogs ($P < 0.05$). There was no significant difference between the latter two groups. This relatively high concentration probably reflects the low concentration of the individual immunoreactive polypeptides within the crude *D. farinae* extract. These results suggest that circulating T-cell responses are specific to dogs sensitised to *D. farinae*, confirming a role for allergen-specific T-cells in the pathogenesis of canine atopic dermatitis. The association of *D. farinae* specific PBMC proliferation with skin test reactivity implies that these cells are supporting *D. farinae* specific IgE production (Lydyard and Grossi, 1998).

A few *D. farinae* negative atopic dogs did have appreciable PBMC responses to *D. farinae*. This may have been due to false negative skin tests (due to technique, degraded allergen, anti-inflammatory medication, etc.) or false positive PBMC proliferation assays. An alternative hypothesis is that these dogs have a predominantly cell-mediated immune response to *D. farinae* with circulating cells of a T_{H1}-type phenotype. These have been

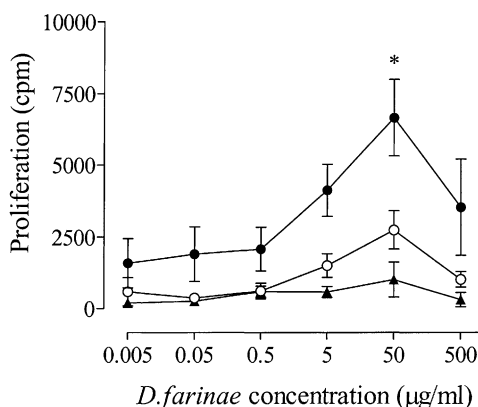


Fig. 1. PBMC responses to *D. farinae* in atopic and healthy dogs: (●) dogs with atopic dermatitis skin test positive to *D. farinae* ($n = 10$); (○) dogs with atopic dermatitis skin test negative to *D. farinae* ($n = 7$); (▲) healthy dogs ($n = 10$); (*) $P < 0.05$; bar, S.E.M.; cpm, counts per min (expressed as, test wells-background – resting wells-background).

associated with protective immunity in humans (Maggi, 1998), but it is not known if this is the case in dogs. The distribution of clinical signs and epidermal focusing of Langerhans' cells and lymphocytes in canine atopic dermatitis is evidence of percutaneous allergen uptake (Olivry et al., 1997). It is possible that some individuals develop a type IV hypersensitivity reaction akin to contact dermatitis (Kalish and Askenase, 1999), rather than an IgE response. Late phase inflammatory responses and chronic lesions in human atopic dermatitis are also dominated by T_H1 -type cytokines (Thepen et al., 1996). It is unclear, if these responses are allergen-specific or not (Cooper, 1994), but if cell-mediated immunity plays role in the pathogenesis of atopic dermatitis, some individuals could have allergic atopic dermatitis without the production of serum antibodies. Diagnostic tests such as intradermal or skin prick tests and serology might not, therefore, be appropriate for all patients.

There was very little response to *D. farinae* in PBMCs from healthy dogs, even though most individuals are exposed to ubiquitous allergens such as the house dust mites. A low level of reactivity is also reported in PBMCs from healthy humans (Horneff et al., 1996). However, PBMC responses to Der p1 have been detected in both atopic and healthy humans using serum free media (Upham et al., 1995; Kawamura et al., 1998). This suggests that the serum components selectively inhibit proliferation of PBMCs from healthy individuals. No serum free medium has been evaluated for canine PBMC cultures, so it remains to be seen if widespread responses to ubiquitous allergens occur in dogs.

Poor responses in allergen-specific PBMC proliferation assays may also be due to peripheral tolerance. It is thought that allergen-specific T-regulatory cells producing IL-10 and TGF β inhibit inflammatory responses in healthy individuals (Koulis and Robinson, 2000; Muraille and Leo, 1999). These cells may also limit in vitro PBMC responses to allergens.

The protocols used in this study, yielded a mean population of 88% PBMCs with greater than 95% viability. Canine PBMC populations vary between individuals, but are

Table 1
PBMC responses to ConA and vaccine antigens

	Mean (S.D.) proliferation (cpm) ^a			
	Resting	ConA	<i>Bordetella</i>	Parvovirus
<i>Dermatophagoides farinae</i> positive atopic dogs (n = 10)	1909 (1848)	49935 (23178)	5736 (6535)	3975 (4485)
<i>Dermatophagoides farinae</i> negative atopic dogs (n = 7)	1144 (883)	38830 (20880)	2541 (3564)	3629 (3704)
Healthy dogs (n = 10)	1176 (1314)	45587 (32037)	2901 (4418)	2979 (3787)

^a cpm: counts per min (for ConA, *Bordetella* and Parvovirus expressed as, test wells-background – resting wells-background).

approximately 15–30% B-cells, 25–50% CD4⁺ T-cells and 20–40% CD8⁺ T-cells (Kristensen et al., 1982; Rivas et al., 1995). Although both B- and T-cells could contribute to cell proliferation, B-cells require interaction with activated CD4⁺ T_H-cells (Lydyard and Grossi, 1998). Most of the response to *D. farinae* allergens seen in this study should be due to CD4⁺ cells, particularly as exogenous antigens are usually presented by antigen presenting cells in the context of an MHC class II molecule to CD4⁺ cells (Kalish and Askenase, 1999). However, exogenous antigens can also be presented with MHC class I molecules to CD8⁺ cells (Kalish and Askenase, 1999). Furthermore, CD8⁺ cells can provide help for B-cell activation and differentiation in certain circumstances (Kemeny, 1998), so it is difficult to make conclusions about the relative contributions of allergen-specific CD8⁺ and CD4⁺ responses in PBMC proliferation assays.

There were no significant differences in the response of PBMCs isolated from the atopic and healthy dogs to any of the control stimuli (see Table 1). All the PBMC cultures in this study responded to ConA, which closely mimics antigen specific T-cell proliferation (Lydyard and Grossi, 1998). This demonstrates that PBMCs from all three groups of dogs were capable of quantitatively similar responses. Vaccine antigens were used to demonstrate that the PBMC cultures were capable of specific recall responses. Natural exposure to *B. bronchiseptica* is common in the domestic and kennelled dogs, but natural and vaccine immunity wanes after 6 months (Ford and Vaden, 1990). Infection with parvovirus is uncommon, but natural and vaccine immunity is robust for at least 12 months (Hoskins, 1990). Despite this, only 8/10 *D. farinae* positive, 3/7 *D. farinae* negative and 4/10 healthy dogs had a response greater than 1000 cpm above resting to parvovirus, and only 5/10 *D. farinae* positive, 6/7 *D. farinae* negative and 6/10 healthy dogs responded to *B. bronchiseptica*. This may be due to sub-optimal culture conditions or cytopathic effects, although PBMCs that did respond did so very well. No reliable and universal recall antigen has been established in dogs (Corato et al., 1997) and the effects of vaccination and natural exposure on PBMC responses are unclear.

D. farinae, despite its low prevalence, is one of the most common skin test reactions in the UK (Sture et al., 1995). However, a recent study speculated that an apparent low prevalence of *D. farinae* could be due to seasonal fluctuations, as equal levels of Der f1 and Der p1 were present in the house dust (Sopelete et al., 2000). Der f15, a major *D. farinae* allergen recognised by dogs, is heavily glycosylated (McCall et al., 2001), which may

enhance its allergenicity (Huby et al., 2000). Despite this, a recent study found that differences in the frequency with which serum IgE from atopic dogs bound to Western blots of *D. farinae* and *D. pteronyssinus* were restricted to a single allergen source (Nuttall et al., 2001). Further work to evaluate the house dust mite fauna and standardise allergen extracts is necessary to define the relative importance of these mites in canine atopic dermatitis.

In conclusion, this study has shown that PBMC responses to *D. farinae* allergens are specific to *D. farinae* sensitised atopic dogs. This confirms that allergen-specific circulating T-cells are involved in the pathogenesis of canine atopic dermatitis. Investigating the cytokine responses induced by *D. farinae* in PBMC cultures should shed further light on the role of T_{H1}/T_{H2} polarisation and peripheral tolerance in atopic and healthy dogs.

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