

Peripheral blood mononuclear cell responses to major and minor *Dermatophagoides* allergens in canine atopic dermatitis

T.J. Nuttall^{a,*}, A.D. Pemberton^a, J.R. Lamb^b, P.B. Hill^a

^aEaster Bush Veterinary Centre, University of Edinburgh Hospital for Small Animals,
Easter Bush, Midlothian EH25 9RG, UK

^bImmunobiology Group and Respiratory Medicine Unit, MRC Centre for Inflammation Research,
University of Edinburgh of Medical School, Teviot Place, Edinburgh, Midlothian, UK

Received 17 May 2001; received in revised form 29 August 2001; accepted 5 October 2001

Abstract

Atopic dermatitis is a chronic inflammatory and pruritic skin disease commonly seen in dogs and humans. Most cases involve hypersensitivity to the house dust mites (HDM) *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*. Human atopic dermatitis is associated with the HDM derived allergens Der f 1 and 2, and Der p 1 and 2. Serological data, however, suggest that a 98/104 kD protein is the most important allergen in dogs with atopic dermatitis. The aim of this study was to characterise the specificity of circulating T-cells in canine atopic dermatitis for HDM derived allergens. Peripheral blood mononuclear cells (PBMCs) from dogs with atopic dermatitis that were skin test positive for *D. farinae* and *D. pteronyssinus* were cultured with crude extracts of *D. farinae*, *D. pteronyssinus* and *D. microceras*, a 98/104 kD allergen purified from *D. farinae*, Der f 1 and Der f 2. There was significantly greater responsiveness of PBMCs to the *D. farinae* and *D. pteronyssinus* extracts compared to the *D. microceras* extract, and similarly to the purified 98/104 kD allergen compared to Der f 1 and Der f 2. The close association between serological findings and PBMC proliferation implies that the 98/104 kD HDM protein is a major target of immune recognition and that T-cells also participate in the pathogenesis of canine atopic dermatitis by supporting IgE production. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Canine atopic dermatitis; Dog; T-cells; House dust mites; *Dermatophagoides farinae*

* Corresponding author. Tel.: +44-131-650-7678; fax: +44-131-650-7652.
E-mail address: timn@vet.ed.ac.uk (T.J. Nuttall).

1. Introduction

Atopic dermatitis is a universal, chronic inflammatory skin disease of humans (Rothe and Grant-Kels, 1996) and dogs (Scott et al., 1995), characterised by positive skin tests and specific serum IgE to *Dermatophagoides* species house dust mites (HDMs) (Halbert et al., 1995; Sture et al., 1995). *Dermatophagoides pteronyssinus* is the most common species in the UK (Thomas and Smith, 1999), although skin test reactions to the closely related species *Dermatophagoides farinae* are more frequent in atopic dogs (Sture et al., 1995). A third species, *D. microceras*, is rare in the UK (O'Hehir, 1988).

The most important *Dermatophagoides* allergens relevant to humans are the group 1 and 2 allergens (Thomas and Smith, 1999), Der p 1 and 2 from *D. pteronyssinus*, and Der f 1 and 2 from *D. farinae*. High IgE titres to these allergens are present in 70–80% of atopic human sera, whereas variable titres to other groups are found in only 40–50% of sera (Thomas and Smith, 1999). However, immunoblotting studies with canine sera found that a 98/104 kD major allergen was common to *D. farinae* and *D. pteronyssinus*, whereas group 1 and 2 allergens were of minor importance (Noli et al., 1996; McCall et al., 2001; Shaw, 2000; Nuttall et al., 2001a). In contrast, another investigation suggested that both Der p 1 and 2, and Der f 1 and 2 were major allergens (Masuda et al., 1999).

Canine atopic dermatitis has been traditionally regarded as an IgE/mast cell mediated disease (Scott et al., 1995) and investigations of PBMC responses to allergens are limited. We have recently shown that PBMCs isolated from *D. farinae* sensitised atopic dogs proliferated in response to a crude extract of *D. farinae*, whereas PBMCs from skin test negative atopic dogs or healthy dogs showed little response (Nuttall et al., 2001b). In another, unpublished, study PBMCs isolated from dogs with atopic dermatitis responded to both a crude *D. pteronyssinus* extract and the 98/104 kD major allergen, but not Der p 1 or Der p 2 (Shaw, 2000).

The aim of this study, therefore, was to further characterise the allergen specificity of circulating T-cells in canine atopic dermatitis by investigating the proliferative response of PBMCs isolated from dogs with atopic dermatitis to group 1 and 2 allergens and the 98/104 kD major allergen purified from *D. farinae*, as well as to crude extracts of *D. farinae*, *D. pteronyssinus* and *D. microceras*.

2. Materials and methods

2.1. *Dermatophagoides* extracts

Freeze dried crude *D. farinae* and *D. pteronyssinus* extracts were obtained from Greer Laboratories, Lenoir, USA (Greer), and a freeze dried crude *D. microceras* extract from ALK-Abelló, Hørsholm, Denmark (ALK). Freeze dried purified Der f 1 and Der f 2 extracts were obtained from ALK-Abelló.

The 98/104 kD *D. farinae* allergen was purified from the Greer *D. farinae* extract by high-pressure liquid chromatography (Waters 486/600S/626 system; Waters-Millipore, Macclesfield, UK). Although it co-purified with a 128 kD protein, Western blotting and probing with a positive control serum confirmed specificity for the 98/104 kD allergen

(data not shown). All the extracts were diluted to 1 mg/ml in sterile PBS (BCA protein assay; Pierce Laboratories, Rockford, USA) and stored at -20°C .

2.2. 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 possibility of 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 Greer *D. farinae* and *D. pteronyssinus* extracts, 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). Negative control samples were taken from three 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.

2.3. Sample collection

Blood samples were collected into 1.2% tri-sodium citrate by jugular venepuncture from atopic dogs with positive intradermal skin test reactions to *D. farinae* and *D. pteronyssinus* ($n = 13$). Blood samples from healthy dogs ($n = 3$) 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). Twenty millilitres 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 440g 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 250g for 10 min at 4°C . The cell pellet was re-suspended in 9 ml of sterile distilled water for 30 s before adding 1 ml of $10\times$ PBS. The cell suspension was then washed in a $3\times$ volume of chilled citrated PBS and centrifuged at 200g 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 μM 2-mercaptoethanol (Life Technologies), 100 IU/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 0.25 $\mu\text{g/ml}$ amphotericin B (100 $\times$ antibiotic-antimycotic solution; Sigma) and 1% non-essential

amino acids (Life Technologies) (hereafter referred to as complete medium). One hundred microlitres of the cell suspension was diluted 1:1 with trypan blue (Sigma). 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 crude *D. farinae*, *D. pteronyssinus* and *D. microceras* extracts were diluted to 100 µg/ml, and the 98/104 kD *D. farinae* major allergen, Der f 1 and Der f 2 to 10 µg/ml in complete medium. One hundred microlitres of each extract was added to the appropriate wells of a 96 well U-bottomed tissue culture plate (Nunclon Microwell; Fisher Scientific, Loughborough, UK). Wells with 100 µl of 1.0 µg/ml ConA (Sigma), 1/10 *Bordetella bronchiseptica* (Intrac[®]; Schering-Plough Animal Health, Uxbridge, UK) and 1/10,000 canine parvovirus vaccine antigens (Parvo C[®]; Intervet; Milton Keynes, UK) diluted in complete medium were included as positive controls. Wells with 100 µl of complete medium were included as negative control resting wells. One hundred microlitres of the cell suspension (i.e. 1×10^5 cells) was added to each well. Wells with 200 µl 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. 0.5 µCi methyl-³H thymidine (Amersham-Pharmacia Biotech, Little Chalfont, UK) was added to each well for the final 6 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, CA, USA), air dried, sealed in counting bags with 4 ml scintillant (Optiscint HiSafe; Wallace, Turku, Finland) and the activity of each well recorded as counts per minute (cpm) using a beta-counter (Microbeta Plus; Wallace).

2.6. Data analysis

Results were expressed as the mean cpm per triplicate wells. Mean background cpm (wells with medium only; range 0–36 cpm) was subtracted from all other wells. After ensuring data were normally distributed, analyses of variance with Tukey's post tests were used to compare the response of PBMCs isolated from atopic dogs to the different crude and purified allergens (Instat; Graphpad, San Diego, USA). Significance was set at $p < 0.05$.

3. Results and discussion

This study demonstrates that T-cells specific for the 98/104 kD allergen purified from *D. farinae* dominate the peripheral repertoire reactive with HDM in canine atopic dermatitis. PBMCs isolated from *D. farinae* sensitised atopic dogs had a significantly greater response to the 98/104 kD *D. farinae* major allergen than to either Der f 1 or 2 (Table 1, Fig. 1). There was no significant difference in the responses to Der f 1 and 2. This is in contrast to humans, where T-cells from 80% of atopic patients respond to group 1 and 2

Table 1

In vitro responses of PBMCs isolated from dogs with atopic dermatitis and healthy dogs^a

Stimulus	Mean cpm (S.D.; number of dogs)	
	Atopic dermatitis	Healthy dogs
<i>D. farinae</i>	11850 (4787; 13)	2431 (3392; 3)
<i>D. pteronyssinus</i>	13861 (9085; 13)	2961 (2922; 3)
<i>D. microceras</i>	4208 (3774; 13)	1834 (2473; 3)
98/104 kD <i>D. farinae</i> allergen	8893 (2132; 7)	1056 (1669; 3)
Der f 1	4337 (2154; 11)	1551 (2576; 3)
Der f 2	2394 (1593; 11)	1189 (2011; 3)
ConA	56040 (17566; 13)	29455 (11424; 3)
<i>B. bronchiseptica</i>	4114 (3310; 13)	1102 (510; 3)
Canine parvovirus	3344 (4422; 13)	856 (390; 3)
Resting wells	2562 (1618; 13)	2101 (2013; 3)

^a S.D.: standard deviation; cpm: counts per minute, expressed as mean per triplicate well—background.

Dermatophagoides allergens (Thomas and Smith, 1999), but similar to an unpublished study in which canine PBMCs responded to the 98/104 kD allergen purified from *D. pteronyssinus*, but not Der p 1 or 2 (Shaw, 2000). These results confirm that the 98/104 kD protein is a major *Dermatophagoides* allergen in canine atopic dermatitis, whereas group 1 and 2 allergens are of minor importance. Furthermore, correlation of T-cell responses with IgE levels implies that they participate in the pathogenesis of canine atopic dermatitis by supporting specific IgE production.

It is surprising that different *Dermatophagoides* allergens are important in human and canine atopic dermatitis, as their allergenic properties are due to their biological activities (Huby et al., 2000). In particular, Der p 1 degrades tight junctions, cleaves α 1-antitrypsin, CD23 and CD25, which directly activates mast cells and basophils, inhibits T_H1

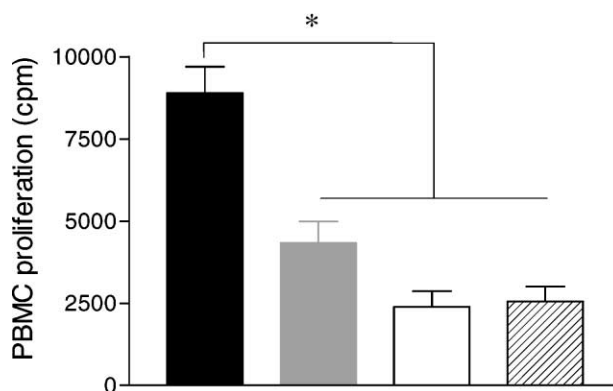


Fig. 1. Responses of PBMCs isolated from dogs with atopic dermatitis to purified *D. farinae* allergens: (■) 98 kD allergen ($n = 7$ dogs); (■) Der f 1 ($n = 11$ dogs); (□) Der f 2 ($n = 11$ dogs); (▨) resting cells ($n = 11$ dogs); bar: S.E.M.; *: $p < 0.05$; cpm: counts per minute, expressed as mean cpm per triplicate wells—background.

differentiation and increases epithelial permeability, protease activity, IgE production and inflammatory mediator release (Schulz et al., 1999; Shakib and Gough, 2000; Wan et al., 2000). However, their in vivo activity could be substrate and species specific. It is unknown if the 98/104 kD allergen is biologically active, although it is glycosylated (McCall et al., 2001), which enhances IgE binding and uptake by APCs (Huby et al., 2000). Furthermore, unlike B-cells, T-cells recognise short sequences of 10–15 amino acids associated with class II MHC molecules (Renz, 1995). Processing of allergen by APCs can profoundly influence the subsequent immune response (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.

All the dogs with a clinical diagnosis of atopic dermatitis in this study had positive skin tests to *D. farinae* and *D. pteronyssinus*. There were also isolated reactions to a variety of other mite, epithelial, pollen and mould allergens. PBMCs isolated from these dogs had significantly greater responses to the crude *D. farinae* and *D. pteronyssinus* extracts than to the crude *D. microceras* extract (Table 1, Fig. 2). This confirms an earlier study (Nuttall et al., 2001a) demonstrating that far fewer atopic canine sera recognised *D. microceras* compared to *D. farinae* and *D. pteronyssinus*. These results do not discriminate between specific T-cell epitopes on individual *D. farinae* and *D. pteronyssinus* allergens or to cross-reacting epitopes present only in these two species. However, *D. farinae* and *D. pteronyssinus* allergens are 80–90% homologous (Thomas et al., 1998) and cross-inhibition with canine sera has shown that their allergens strongly cross-react (Masuda et al., 1999).

PBMCs isolated from the healthy dogs did not respond to the *Dermatophagoides* allergens despite comparable responses to the control stimuli (Table 1), suggesting that the differences between the extracts were not due to non-specific mitogenic activity. Inhibition by any of the extracts is also unlikely as some atopic PBMCs did respond and all are routinely used in other studies (G. Nordskov Hansen; ALK, Hørsholm, Denmark, personal communication). Vaccine antigens were used to demonstrate that the PBMCs were capable of specific recall responses. Exposure to *B. bronchiseptica* is common in domestic and kennelled dogs, but natural and vaccine immunity wanes after 6 months (Ford and Vaden,

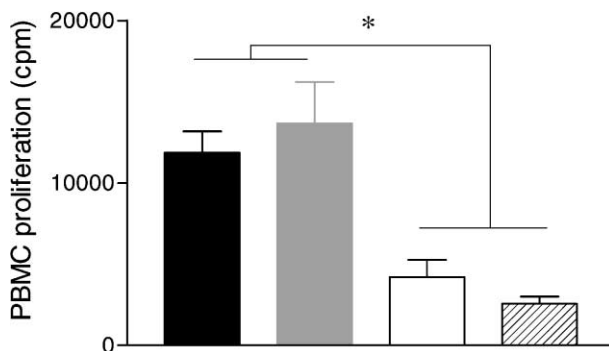


Fig. 2. Responses of PBMCs isolated from dogs with atopic dermatitis to crude *Dermatophagoides* extracts: (■) *D. farinae* (n = 13 dogs); (▨) *D. pteronyssinus* (n = 13 dogs); (□) *D. microceras* (n = 13 dogs); (▤) resting cells (n = 13 dogs); bar: S.E.M.; *: $p < 0.05$; cpm: counts per minute, expressed as mean cpm per triplicate wells—background.

1990). Parvovirus infections are uncommon, but natural and vaccine immunity is robust for at least 12 months (Hoskins, 1990). Despite this, the response was very variable (Table 1). 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.

The protocols used in this study yielded a mean population of 79% PBMCs with greater than 95% viability. Canine PBMC populations vary between individuals, but are 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-cells (Lydyard and Grossi, 1998). Most of the response to *Dermatophagoides* allergens seen in this study is likely to be due to CD4⁺ cells, particularly as exogenous antigens are usually presented by APCs with 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.

In conclusion, this study has shown that PBMC responses to *D. farinae* allergens are specific to the 98/104 kD major allergen identified in immunoblotting studies. This confirms that allergen specific circulating T-cells are involved in the pathogenesis of canine atopic dermatitis. Investigating the responses to overlapping peptides and the biological activity of this allergen should shed light on the mechanism of sensitisation.

Acknowledgements

The authors are grateful for the help and assistance shown by Dr. K.L. Thoday, Mr. A.W. Carter and Mr. P. Fosythe of the Dermatology Service at the University of Edinburgh Hospital for Small Animals, and Dr. G. Hoyne, Dr. S. Howie, Dr. N. Savage and Ms. G. Hall of the Immunobiology Group and Respiratory Medicine Unit, MRC Centre for Inflammation Research. The authors are also grateful to ALK-Ab  llo for supplying the Der f 1 and Der f 2 extracts. This study was funded by The Wellcome Trust.

References

- Corato, A., Shen, C.-R., Mazza, G., Barker, R.N., Day, M.J., 1997. Proliferative responses of peripheral blood mononuclear cells from normal dogs with autoimmune haemolytic anaemia to red blood cell antigens. *Vet. Immunol. Immunopathol.* 59, 191–204.
- Ford, R.B., Vaden, S.L., 1990. Canine infectious tracheobronchitis. In: Greene, C.E. (Ed.), *Infectious Diseases of the Dog and Cat*. Saunders, Philadelphia, pp. 33–38.
- Halbert, A.R., Weston, W.L., Morelli, J.G., 1995. Atopic dermatitis: is it an allergic disease? *J. Am. Acad. Dermatol.* 33, 1008–1018.
- Hoskins, J.D., 1990. Canine viral enteritis. In: Greene, C.E. (Ed.), *Infectious Diseases of the Dog and Cat*. Saunders, Philadelphia, pp. 40–49.
- Huby, R.D., Dearman, R.J., Kimber, I., 2000. Why are some proteins allergens? *Toxicol. Sci.* 55, 235–246.

- Kalish, R.S., Askenase, P.W., 1999. Molecular mechanisms of CD8⁺ T cell-mediated delayed hypersensitivity: implications for allergies, asthma and autoimmunity. *J. All. Clin. Immunol.* 103, 192–199.
- Kemeny, D.M., 1998. CD8⁺ T cells in atopic disease. *Curr. Opin. Immunol.* 10, 628–633.
- Kristensen, F., Kristensen, B., Lazary, S., 1982. The lymphocyte stimulation test in veterinary immunology. *Vet. Immunol. Immunopathol.* 3, 203–277.
- Lydyard, P., Grossi, C., 1998. Cells involved in the immune response. In: Roitt, I., Brostoff, J., Male, D. (Eds.), *Immunology*, 5th Edition. Mosby, London, pp. 14–30.
- Maekawa, Y., Himeno, K., Hiroyuki, I., Hisaeda, H., Sakai, T., Dainichi, T., Asao, T., Good, R.A., Katunuma, N., 1998. Switch of T-cell differentiation from Th2 to Th1 by treatment with cathepsin B inhibitor in experimental leishmaniasis. *J. Immunol.* 161, 2120–2127.
- Masuda, K., Tsujimoto, H., Fujiwara, S., Kurata, K., Hasegawa, A., Yasueda, H., Yamashita, K., DeBoer, D.J., deWeck, A.L., Sakaguchi, M., 1999. IgE sensitivity and cross-reactivity to crude and purified mite allergens (Der f 1, Der f 2, Der p 1, Der p 2) in atopic dogs sensitive to *Dermatophagoides* mite allergens. *Vet. Immunol. Immunopathol.* 72, 303–313.
- McCall, C., Hunter, S., Weber, E., Stedman, K., Hillier, A., Bozic, C., Rivoire, B., Olivry, T., 2001. Characterization and cloning of a major high molecular weight house dust mite allergen (Der f 15) for dogs. *Vet. Immunol. Immunopathol.* 78, 231–247.
- Noli, C., Bernadina, W.E., Willemse, T., 1996. The significance of reactions to purified fractions of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* in canine atopic dermatitis. *Vet. Immunol. Immunopathol.* 52, 147–157.
- Nuttall, T.J., Thoday, K.L., van den Broek, A.H.M., Jackson, H.A., Sture, G.H., Halliwell, R.E.W., 1998. Retrospective survey of allergen immunotherapy in canine atopy. *Vet. Rec.* 143, 139–142.
- Nuttall, T.J., Lamb, J.R., Hill, P.B., 2001a. Characterisation of major and minor *Dermatophagoides* allergens in canine atopic dermatitis. *Res. Vet. Sci.* 71, 51–57.
- Nuttall, T.J., Lamb, J.R., Hill, P.B., 2001b. Peripheral blood mononuclear cell responses to *Dermatophagoides farinae* in canine atopic dermatitis. *Vet. Immunol. Immunopathol.* 82, 273–280.
- O'Hehir, R.E., 1988. Polyclonal and monoclonal analysis of the human T lymphocyte response to *Dermatophagoides* species. Ph.D. Thesis. University of London, London, pp. 1–251.
- Renz, H., 1995. The central role of T-cells in allergic sensitisation and IgE regulation. *Exp. Dermatol.* 4, 173–182.
- Rivas, A.L., Kimball, E.S., Quimby, F.W., Gebhard, D., 1995. Functional and phenotypic analysis of in vitro stimulated canine peripheral blood mononuclear cells. *Vet. Immunol. Immunopathol.* 45, 55–71.
- Rothe, M.J., Grant-Kels, J.M., 1996. Atopic dermatitis: an update. *J. Am. Acad. Dermatol.* 35, 1–13.
- Schulz, O., Sewell, H.F., Shakib, F., 1999. The interaction between the dust mite antigen Der p 1 and cell-signalling molecules in amplifying allergic disease. *Clin. Exp. All.* 29, 439–444.
- Scott, D.W., Miller, W.H., Griffin, C., 1995. Immunologic skin disease. In: Muller and Kirk's Small Animal Dermatology. Saunders, Philadelphia, pp. 484–626.
- Shakib, F., Gough, L., 2000. The proteolytic activity of Der p 1 selectively enhances IgE synthesis: a link between allergenicity and cysteine protease activity. *Clin. Exp. All.* 30, 751–752.
- Shaw, S.C., 2000. House dust mite allergens: identification of the allergens of *Dermatophagoides pteronyssinus*. In: Proceedings of the British Veterinary Dermatology Study Group Spring Meeting, Birmingham, pp. 19–22.
- Sture, G.H., Halliwell, R.E.W., Thoday, K.L., van den Broek, A.H.M., Henfrey, J.I., Lloyd, D.H., Mason, I.S., Ferguson, E., 1995. Canine atopic dermatitis: the prevalence of positive intradermal skin tests at two sites in the north and south of Great Britain. *Vet. Immunol. Immunopathol.* 44, 293–308.
- Thomas, W.R., Smith, W., 1999. Towards defining the full spectrum of important house dust mite allergens. *Clin. Exp. All.* 29, 1583–1587.
- Thomas, W.R., Smith, W., Hales, B.J., 1998. House dust mite allergen characterisation: implications for T-cell responses and immunotherapy. *Int. Arch. All. Immunol.* 115, 9–14.
- Wan, H., Winton, H.L., Soeller, C., Gruenert, D.C., Thompson, P.J., Cannell, M.B., Stewart, G.A., Garrod, D.R., Robinson, C., 2000. Quantitative structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p 1. *Clin. Exp. All.* 30, 685–698.
- Willemse, T., 1986. Atopic skin disease: a review and a reconsideration of diagnostic criteria. *J. Small Anim. Prac.* 27, 771–778.