The immunopathogenesis of canine atopic dermatitis

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Declaration

This thesis describes work performed by the author under the supervision of Professor Jonathan Lamb and Dr. Peter Hill for the degree of PhD. The material is wholly the work of the author whilst recognising the advice and training received from those persons mentioned in the acknowledgements. Exceptions to this are the diagnostic investigations, intradermal allergen tests and microbiological examinations carried out by the clinician in charge of each case at the University of Edinburgh Hospital for Small Animals; allergen specific IgE ELISAs performed by Axiom Laboratories, Teignmouth, Devon; processing and staining of the histopathological skin sections undertaken by the University of Edinburgh Royal (Dick) School of Veterinary Studies Department of Veterinary Pathology; and high performance liquid chromatography separation of the *Dermatophagoides farinae* allergens by Dr. Alan Pemberton of the University of Edinburgh Royal (Dick) School of Veterinary Studies Department of Veterinary Clinical Studies.

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Summary

Atopic dermatitis (AD) is a chronic inflammatory and pruritic skin disease commonly seen in dogs and humans. Most cases involve hypersensitivity to the house dust mites *Dermatophagoides farinae* and *D. pteronyssinus*. This study has shown that *Dermatophagoides* specific IgE and peripheral blood mononuclear cell (PBMC) responses were largely restricted to skin test positive atopic dogs. Furthermore, immunoblotting and PBMC proliferation studies demonstrated that the major target of immune recognition is a 98/104kD protein, rather than the low molecular weight group 1 and 2 proteins important in humans. The close association between serological findings and PBMC proliferation implies that T-cells participate in the pathogenesis of canine AD by supporting IgE production. Human AD is associated with T-helper 2 (T_{H2}) type responses, although T_{H1} cytokines are present in chronic lesions. In contrast, tolerance in healthy individuals is mediated by regulatory T-cells. Using semi-quantitative reverse transcriptase-polymerase chain reactions, this study found that canine AD is associated with over-production of the T_{H2} cytokine interleukin (IL)-4, whilst tolerance in healthy individuals is associated with the immunosuppressive cytokines transforming growth factor beta and IL-10. Higher levels of the T_{H1} cytokines interferon gamma, tumour necrosis factor alpha and IL-2 seen in lesional compared to non-lesional and healthy skin could be induced by subsequent self-trauma and secondary infections. These results characterise the first spontaneously occurring animal model of human AD.

List of abbreviations

APC	Antigen presenting cell
BMMC	Bone marrow derived mast cells
MC_C	Chymase mast cell
ConA	Concanavalin A
cpm	counts per minute
CLA	Cutaneous lymphocye antigen
DLA	Dog leucocyte antigen
D	Dorsal
ELISA	Enzyme linked immunosorbant assay
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage colony stimulating factor
FceRI	High affinity IgE receptor
HLA	Human leucocyte antigen
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ICAM	Intercellular adhesion molecule
IFNγ	Interferon gamma
IL	Interleukin
IDT	Intradermal test
FcεRII	Low affinity IgE receptor
MHC	Major histocompatability complex
mRNA	messenger RNA
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PG	Prostaglandin
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sd	Standard deviation
sem	Standard error of the mean
SE	Staphylococcal enterotoxin
SCF	Stem cell factor
T_C	T cytotoxic cells
TCR	T-cell receptor
T_H	T-helper cell
TARC	Thymus and activation regulated chemokine
TSST	Toxic shock syndrome toxin
TGFβ	Transforming growth factor beta
MC_{TC}	Tryptase-chymase mast cell
TNFα	Tumour necrosis alpha
VCAM	Vascular cell adhesion molecule
V	Ventral

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1. Introduction

1.1 Introduction to human and canine atopic dermatitis

Atopic dermatitis is a chronic, relapsing and pruritic inflammatory skin disease universally recognised in humans and dogs. In both the human (Rothe and Grant-Kels 1996) and veterinary literature (Scott and others 2001a) the cause is defined as an inherited predisposition to develop IgE antibodies specific for environmental allergens that are non-pathogenic in healthy individuals.

1.1.1 Atopic dermatitis and allergic sensitisation

The persistent generation of IgE antibodies to environmental allergens is characteristic of type I hypersensitivity diseases (Brostoff and Hall 1998). Elevated levels of cutaneous and circulating allergen-specific IgE can be demonstrated in most cases of human and canine atopic dermatitis by serology, skin tests and basophil release tests (Leung 2000; Scott and others 2001a). Furthermore, exacerbations and ameliorations of human atopic dermatitis often follow exposure to environmental allergens (Wistokat-Wulfing and others 1999).

There are, however, human (Halbert and others 1995; Akdis and others 1999a) and canine (Scott and others 2001a) cases with no allergen-specific IgE or skin test reactivity that fulfil the clinical diagnostic requirements for atopic dermatitis. In humans, it has been postulated that there are two forms of atopic dermatitis - an allergic (extrinsic) form with allergen specific IgE, mast cell reactivity and eosinophil rich cutaneous infiltrates; and a non-allergic (intrinsic) form that is not associated with IgE, mast cell reactivity or eosinophils (Halbert and others 1995; Hanifin 1996; Akdis and

others 1999a; Tanaka and others 1999). T-cells dominate the cutaneous inflammatory infiltrate in both forms, but allergen specific T-cells are present only in the allergic form (Akdis and others 1999a). It is unclear if there are two forms of canine atopic dermatitis, as the clinical and histopathological features of allergen reactive and non-reactive dogs have not yet been studied in detail. However, recent reports speculate that both an atopic constitution and altered skin reactivity are necessary for IgE-mediated atopic dermatitis (Hyland 2001; Sinke and others 2002). Allergen reactive healthy individuals could, therefore, have an atopic constitution without altered skin reactivity, whereas non-IgE mediated atopic dermatitis could be associated with altered skin reactivity without an atopic constitution.

Possible pathogenic mechanisms for non-allergic atopic dermatitis include reduced δ6-desaturase activity, arachidonic acid and di-homo gamma linoleic acid levels, which have been detected in some atopic humans (Hanifin 1996). Furthermore, abnormal lipid metabolism and bacterial ceramidase activity may decrease epidermal ceramide levels (Ohnishi and others 1999; Hara and others 2000). This could impair the cutaneous barrier and enhance inflammatory reactions to irritants, microorganisms and possibly allergens (Leung 2000).

The role of the epidermal lipid barrier in canine atopic dermatitis is unclear. Abnormalities in δ6 and δ5-desaturase activity, decreased fat absorption and ultrastructural abnormalities in the epidermal lipid barrier have been reported in atopic dogs (Horrobin and others 1990; van den Broek and Simpson 1990; Scott and others 1997; Taugbol and others 1998; Olivry and others 2000), but their pathogenic significance is unclear.

1.1.2 The incidence of allergic diseases in humans and dogs

Despite the pathogenetic heterogeneity, most cases of human (Leung 2000) and canine (Scott and others 2001a) atopic dermatitis appear to be a type I hypersensitivity. The incidence of human allergic disease in developed societies has increased dramatically in the last 30 years (Holt 1998). 10-30% of children now suffer from allergies (Hopkin 1997; Thomas and others 1998a; Leung 2000) and 30-35% of all individuals can be affected at some time in their lives (Holt and Macaubas 1997).

There is little robust epidemiological data for canine atopic dermatitis, but one standard text estimates that up to 10% of all dogs are affected (Scott and others 2001a). A recent review found that between 3 and 30% of all cases seen in private and university teaching hospitals were diagnosed with atopic dermatitis (Hillier and Griffin 2001). However, these findings are difficult to interpret as the data varied with geography, the case population (general population, all diseases, dermatological problems etc.), the type of veterinary practice (general, private dermatology or teaching hospital) and diagnostic criteria.

Whether the incidence has increased to the same extent as in humans is debatable, as this period coincides with the development of improved diagnostic techniques in veterinary dermatology. However, dogs have changed from predominantly outdoor working animals to indoor companion animals over the same time-scale and are now exposed to the same environmental influences as humans. Early vaccination, parasite control and antibiotic use have also increased. Canine atopic dermatitis could, therefore, be a valuable spontaneous model for studying the human disease.

1.2 Clinical features of atopic dermatitis

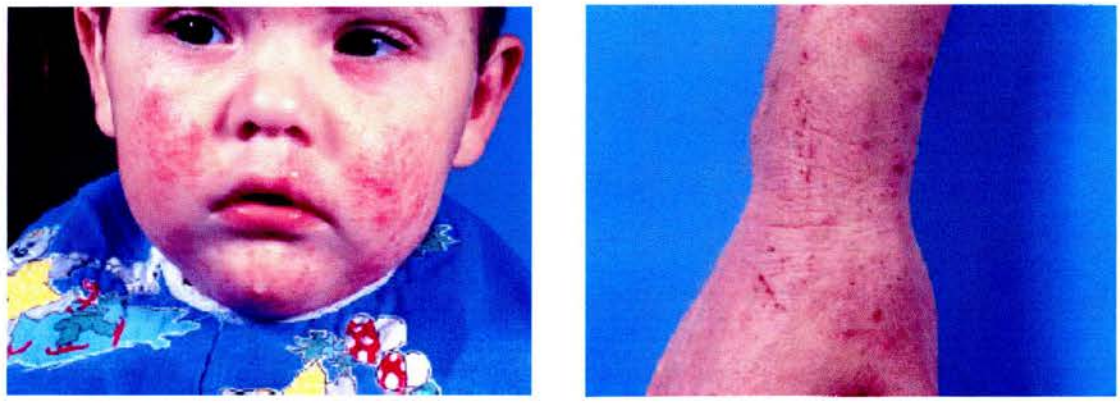
1.2.1 Human atopic dermatitis

Atopic dermatitis is part of an atopic syndrome that includes dermatitis, rhinitis, conjunctivitis and asthma (Rothe and Grant-Kels 1996; Leung 2000). 90% of cases develop in the first five years of life (Leung 2000). The clinical course often results in complete remission by the late teens or early twenties, but clinical signs persist into adulthood in up to 60% of sufferers (Hanifin 1996; Beltrani 1999a). A significant proportion of children with atopic dermatitis will subsequently develop atopic asthma (Leung 2000).

The clinical hallmark of human atopic dermatitis is pruritus. Primary skin lesions are rare and limited to erythema and/or a mild papular eruption with hyperhidrosis or xerosis (Rothe and Grant-Kels 1996). The typical eczematous lesions develop after self-trauma or secondary infection (Beltrani 1999a). Chronic inflammation and self trauma often lead to lichenification of affected skin (Halbert and others 1995; Rothe and Grant-Kels 1996). Human atopic dermatitis typically affects the face, head and neck, flexural limb surfaces and trunk, but can be generalised in severe cases (Halbert and others 1995; Rothe and Grant-Kels 1996) (Figure 1.1). Some cases, particularly in adults, can be focal or localised (Beltrani 1999a).

Allergens commonly implicated in atopic dermatitis include house dust mites, pollens, moulds, insects, foods, epithelia and fibres (Beltrani 1999a; Friedmann 1999). Other provocative factors can be pyschological, contact irritants, hormonal, heat, humidity and perspiration (Rothe and Grant-Kels 1996; Beltrani 1999a; Friedmann 1999). Colonisation of lesional skin with staphylococci and *Malassezia* yeasts is common (Blaher and others 1995; Halbert and others 1995; Herz and others 1998).

Figure 1.1 – Facial atopic dermatitis in an infant (left) and flexural atopic dermatitis affecting the hand and wrist in an adult (right) (Thestrup-Pedersen 2000 by kind permission of Blackwell Science).



Atopic dermatitis can be a very distressing condition. The intense pruritus often disturbs sleep and disrupts normal development and family life, affecting quality of life for both the patient and close family (Blaher and others 1995). The physical signs of severe atopic dermatitis can also be disfiguring with consequent social implications (Koblenzer 1999).

1.2.2 Canine atopic dermatitis

Dogs also spontaneously develop an atopy-like syndrome (Scott and others 2001a). Most cases develop between six months and three years of age (Griffin and DeBoer 2001). Dermatitis is the most common manifestation, accounting for 50% of the dermatology referrals in one centre (Nuttall and others 1998). Conjunctivitis is occasionally seen, but respiratory symptoms are rare (Mason 1995; Griffin and DeBoer 2001; Scott and others 2001a).

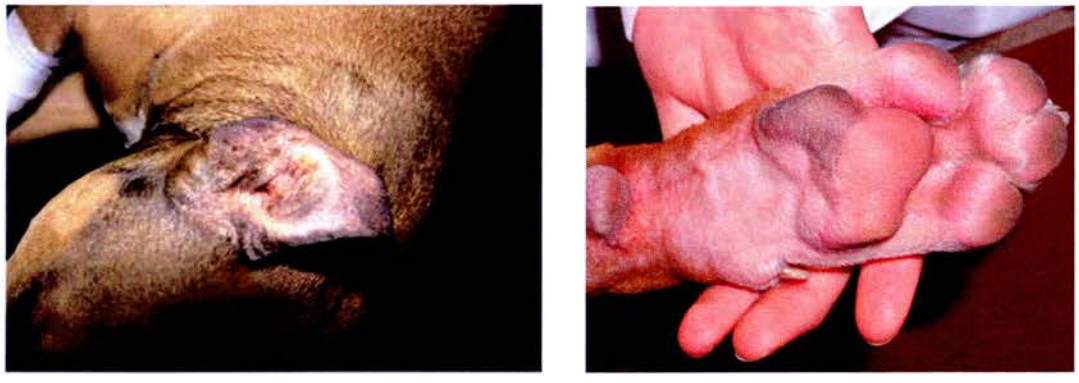
As in humans, pruritus is the most important clinical sign and usually precedes secondary lesions such as erythema or a papular eruption (Mason 1995; Scott and others 2001a). Chronic lesions are often lichenified, hyperpigmented and alopecic, possibly due to repeated self-trauma (Figure 1.2). Microbial colonisation and secondary infection of affected skin with *Staphylococcus intermedius* and *Malassezia pachydermatis* is also common (Scott and others 2001a).

Figure 1.2 – Chronic atopic dermatitis in a West Highland white terrier with extensive excoriation, alopecia, lichenification and hyperpigmentation. This dog also had well-established staphylococcal pyoderma and *Malassezia* dermatitis.

Typically affected sites include the face, ears, flexural surfaces, interdigital skin and ventral body (Mason 1995; Scott and others 2001a) (Figure 1.3). These clinical signs and sites have been suggested as clinical criteria for the diagnosis of atopic dermatitis (Willemse 1986; Prelaud and others 1998). However, caution must be exercised as dogs with other pruritic dermatoses may satisfy these criteria. Furthermore, studies

describing the clinical signs did not use the same inclusion criteria nor necessarily eliminate the possibility of other dermatoses (Griffin and DeBoer 2001).

Figure 1.3 – Diffuse erythema of the ears and interdigital skin of a crossbred dog with atopic dermatitis



Positive reactions to a range of house dust mite, pollen, epidermal, insect and mould allergens are seen with intradermal skin tests or serology for specific IgG and IgE (Sture and others 1995; Day and others 1996; Hill and DeBoer 2001; Scott and others 2001a). Other factors that can contribute to the pathogenesis include food intolerance, poor quality diets, increased temperature and humidity, ectoparasites and endoparasites (Mason 1995; Scott and others 2001a).

Canine atopic dermatitis can be very distressing for both dogs and their owners. The pruritus often interrupts sleep and activity patterns, interfering with the human-animal bond. Pruritus and skin inflammation are also obvious and intrusive clinical signs that owners can readily empathise with.

1.3 Histopathology and immunohistochemistry of atopic dermatitis

1.3.1 Human atopic dermatitis

Histological changes are present in both non-lesional and lesional skin. Compared to healthy skin, there is a sparse perivascular mononuclear, primarily T-cell, infiltrate into non-lesional skin (Leung 1999; Leung 2000). Immunohistochemistry also demonstrates increased expression of T-cell and eosinophil surface markers (Langeveld-Wildschut and others 2000). There can also be mild hyperkeratosis of the stratum corneum (Leung 1999).

The histopathology of acute lesions is dominated by $CD4^+$ T-cell infiltration of the epidermis and superficial dermis. Infiltrating T-cells are predominantly $CD45RO^+CD4^+$ activated memory cells, which express cutaneous lymphocyte antigen (CLA). There is also a marked perivascular dermal infiltrate of mononuclear cells and macrophages with infrequent eosinophils, basophils and neutrophils. Increased numbers of mast cells are occasionally observed, but increased degranulation is frequently seen (Leung 1999; Leung 2000). There is often epidermal spongiosis, acanthosis and exocytosis of T-cells and Langerhans' cells (Herz and others 1998; Leung 1999). Mast cells, basophils, dendritic cells and Langerhans' cells are frequently positive for IgE bound to high (FcεRI) and low (FcεRII/CD23) affinity IgE receptors (Herz and others 1998; Leung 2000).

Chronically inflamed skin exhibits greatly increased hyperkeratosis, acanthosis and spongiosis. Dermal fibrosis may be present in severely affected areas (Herz and others 1998; Leung 1999). There are also increased numbers of IgE^+ Langerhans' cells and dendritic cells in the epidermis and dermis respectively. The dermis is dominated by perivascular to diffuse inflammatory infiltrates of $CD4^+$ and $CD8^+$ CLA^+ lymphocytes,

cytes, monocytes, macrophages, neutrophils, eosinophils and mast cells, which show increased degranulation (Cooper 1994; Hanifin 1996; Leung 1999). The intensity of the infiltration generally correlates with the severity of the skin lesions (Leung 2000).

1.3.2 Canine atopic dermatitis

Like human atopic dermatitis, non-lesional canine atopic skin is characterised by sparse superficial perivascular infiltrates of lymphocytes, monocytes, dendritic cells and mast cells (Nimmo Wilkie and others 1990; Olivry and others 1997). There may also be mild epidermal spongiosis (Olivry and others 1997; Olivry and others 1999a). In lesional skin the degree of epidermal spongosis, acanthosis and hyperkeratosis is related to the severity of the clinical signs (Olivry and others 1997). There is a superficial to deep, perivascular to diffuse dermal infiltration of $CD4^+$ and $CD8^+$ T-cells, monocytes, eosinophils, neutrophils, mast cells and plasma cells that also correlates with the severity of the clinical signs (Olivry and others 1997). The epidermis exhibits increased numbers of IgE^+ Langerhans' cells (Day 1996), $CD4^+$ and, to a lesser (Sinke and others 1997) or greater (Olivry and others 1997) extent, $CD8^+$ T-cells. The number of IgE^+ Langerhans' cells correlates with serum IgE levels (Olivry and others 1999a). Dermal mast cells are also frequently IgE^+ (Olivry and others 1997). $\gamma\delta$ T-cells, which are rare in humans, are seen in the epidermis and dermis in canine atopic dermatitis. Whether they have any activity as effector or regulatory cells is, however, unknown (Olivry and others 1999a).

1.4 Genetic factors in atopic dermatitis

1.4.1 Human atopic dermatitis

A family history is a characteristic feature of human atopic diseases (Rothe and Grant-Kels 1996; Torres-Galván and others 1999). The likelihood of having atopy progressively increases if neither, one or both parents have an atopic history (Rothe and Grant-Kels 1996). There is also an increased incidence in monozygotic compared to dizygotic twins (Rothe and Grant-Kels 1996). These findings suggest that genetic factors are important in developing atopic dermatitis. However, identifying these factors and their mode of inheritance has proved elusive and controversial. It is likely that atopic traits involve several genes, which could be dominant, recessive or display incomplete penetrance (Torres-Galván and others 1999).

Reactivity to pollens has been associated with certain human leucocyte antigen (HLA) haplotypes (Cookson 1998). Increased magnitude, but not frequency, of responses to Der p 2 has been linked to HLA-DQ haplotypes (O'Brien and others 1995), although it has proved difficult to link other allergens with HLA haplotypes (Mitsuyasu and others 1999; Torres-Galván and others 1999). It is also possible that different peptides within an allergen are restricted by different HLA haplotypes, permitting sensitisation in a range of individuals (Cookson 1998).

Other candidates include genes that regulate the expression and activity of cytokines and cytokine receptors, IgE and IgE receptors, antigen processing or T-cells (Leung 1998; Cookson 1998; Torres-Galván and others 1999). Associations to atopic diseases have been made with up regulation of the tumour necrosis factor alpha (TNFα) promoter region and increased signalling by the interleukin (IL)-4 receptor and CD23 (Cookson 1998; Mitsuyasu and others 1999). Serum IgE levels and bronchial re-

sponses have also been linked with particular alleles of chromosome 5q23-31, which contains genes for IL-3, 4, 5, 9, 12 and 13, and chromosome 11q12-13, which governs FcεRI expression (Cookson 1998; Torres-Galván and others 1999). Polymorphisms of the FcεRIβ subunit may amplify allergen/IgE mediated signals, whilst those of the α subunit could increase IgE binding affinity (Bieber 1997). Other links have been made to polymorphisms of chromosome 12q and genes for interferon gamma (IFNγ) and stem cell factor (SCF) (Cookson 1998).

None of these findings have proved consistent, and it is thought that atopic traits could differ between gene pools (Cookson 1998; Leung 1998). However, although the prevalence of atopic dermatitis varies between countries, no link to any ethnic group has been established (Leung 2000). This suggests that environmental factors are more important than genetic traits. Furthermore, the recent rise in the incidence of atopic diseases has been too rapid to explain by genetic mechanisms alone (Polosa 1999). Atopic traits could have evolved to cope with high parasite burdens and could, therefore, be widespread in modern humans (Monticelli and others 1998; Erb 1999).

1.4.2 Canine atopic dermatitis

Well-recognised breed predispositions (e.g. West Highland White Terriers, Labradors and Boxers) suggest that a familial tendency also exists in canine atopic dermatitis (Scott and others 2001a). However, in one study only 13 progeny of 72 affected parents developed atopic dermatitis (Schwartzman 1984). Furthermore, in a Beagle colony with an autosomal dominant high IgE response, the propensity to develop persistent IgE responses and clinical signs depends on early and repeated allergen exposure (de Weck and others 1997).

Dog leucocyte antigen (DLA) haplotypes for class II major histocompatibility complex molecules (MHCII) have recently been identified (Kennedy and others 1999). There is considerable interbreed variation in haplotype and allele frequency, although none identified so far are specific to any one breed. Up to 35% of dogs are homozygous, reflecting the high degree of inbreeding in many breeds (Kennedy and others 1999). The DLA haplotype alleles DRB1*00201 and DRB1*00101 have been associated with polyarthritis and hypothyroidism respectively (Kennedy and others 2001). Serological typing in an earlier study linked a combination of DLA3 and R15 with atopy, but not with serum IgE levels (Vriesendorp and others 1975). Large-scale studies of DLA linkage with clinical atopic dermatitis have yet to be performed.

These findings suggest that, similarly to humans, canine atopic dermatitis is the end result of a complex interaction between genetic traits and environmental influences. However, studies of genetic traits in dogs are limited and need further investigation.

1.5 Atopic dermatitis and house dust mite allergens

1.5.1 House dust mite specific skin tests and serum IgE in atopic dermatitis

Atopic dermatitis may be associated with reactions to a variety of potential allergens including house dust mites, insects, pollens, epithelia, fibres, moulds and foods (Rothe and Grant-Kels 1996; Scott and others 2001a). Recent studies have also described reactions to human skin extracts in human atopic dermatitis (Valenta and others 2000). However, both human (Leung 2000) and canine (Sture and others 1995) atopic dermatitis are predominantly house dust mite mediated.

Human atopic dermatitis is strongly associated with house dust mite exposure. Positive skin tests and allergen specific serum IgE titres are found in 40-80% of patients

(Halbert and others 1995). In one study, 90% of patients with positive skin tests were exposed to house dust mite antigen levels greater than 2μg/g of dust, which is regarded as the threshold for sensitisation (Ricci and others 1999). House dust specific T-cells have been cloned from atopic skin and patch test sites (Leung 2000). House dust mite allergen-specific proliferation of peripheral blood mononuclear cells (PBMCs), furthermore, correlates with atopy patch test results and serum specific IgE levels in atopic, but not healthy, patients (Blaher and others 1995; Horneff and others 1996; Kawamura and others 1998; Wistokat-Wulfing and others 1999).

House dust mite allergens are of similar importance in canine atopic dermatitis. 60-90% of cases in the UK (Sture and others 1995), Japan (Masuda and others 2000), mainland Europe and the US (Scott and others 2001a) have positive skin tests or serum IgE specific for house dust mite allergens. In another study, house dust mite specific proliferation was seen in PBMCs isolated from atopic, but not healthy, dogs (Shaw 2000).

1.5.2 Atopy patch tests and house dust mite allergens

Atopy patch tests, in which there is direct application of allergens to the skin, mimic natural percutaneous allergen exposure. The clinical appearance and histopathology of positive sites closely matches human (Grewe and others 1998) and canine (Sinke and others 1998) atopic dermatitis. Patch tests in humans appear to be IgE mediated, as positive tests correlate with the number of epidermal IgE^+ cells, although not FcεRI expression (Langeveld-Wildschut and others 2000).

Although it is not commonly performed, patch testing with house dust mite allergens is highly specific for human (Grewe and others 1998) and canine (Sinke and others

1998) atopic dermatitis, although an early study in dogs failed to show any association (Frank and McEntee 1995). Despite this, 60-70% of atopic patients have negative patch tests, possibly due to low levels of epidermal IgE or non-percutaneous allergen uptake (Grewe and others 1998). Sensitivity can be increased by scarification of test sites (Wistokat-Wulfing and others 1999), suggesting that the epidermal barrier also blocks allergen uptake. There is no clinical difference between human patients with positive or negative patch tests (Grewe and others 1998).

1.5.3 Increasing exposure to house dust mites

Changes in Western lifestyles may have dramatically increased exposure to indoor allergens like house dust mites. Furthermore, insulation, central heating and reduced ventilation have raised indoor temperatures and humidity, which significantly increases house dust mite populations (Hirsch and others 2000).

1.5.4 The importance of *Dermatophagoides* species house dust mites

The major house dust mite species in temperate areas are *Dermatophagoides farinae* and *D. pteronyssinus*. *D. pteronyssinus* prefers more humid conditions than *D. farinae* and is most common in the UK (Thomas and others 1998a). A third species, *D. microceras*, is rarely found in the UK (O'Hehir 1988). Despite this, skin test reactions to *D. farinae* are more frequent than *D. pteronyssinus* in atopic dogs in the UK (Sture and others 1995). However, having a dog is positively correlated with household Der f 1 levels (Gross and others 2000). Canine squames may be a particularly good substrate for *D. farinae,* or dog owning households may have microclimates favouring this species. A study in an area of Brazil thought to have low numbers of *D. farinae*

found equal levels of indoor Der f 1 and Der p 1 (Sopelete and others 2000). The authors speculate that seasonal population fluctuations could lead to an artificially low estimate of *D. farinae* numbers.

1.5.5 *Dermatophagoides* species allergens

1.5.5.1 Allergens relevant to humans

Dermatophagoides species house dust mites are the most important allergens in human atopic dermatitis (Thomas and Smith 1999). More than 16 different *Dermatophagoides* allergens have been identified (Table 1.1). The most important are the group 1 and 2 allergens Der p 1 and 2, and Der f 1 and 2 from *D. pteronyssinus* and *D. farinae* respectively. High IgE titres to these are present in 70-80% of atopic human sera, whereas variable titres to other groups are found in 40-50% of sera (Le Mao and others 1998; Thomas and others 1998a).

Table 1.1 – Allergens identified in *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*

Mite Species	Allergen Group	Allergen Name	Molecular Weight (kD)	Identity	References
D. pteronyssinus *D. farinae*	1	Der p 1 Der f 1	25-30	Cysteine protease	(O'Hehir and others 1993; Thomas and others 1998a)
D. pteronyssinus *D. farinae*	2	Der p 2 Der f 2	14-20	? Reproductive protein or epithelial secretion involved in ecdysis	(O'Hehir and others 1993; Smith and others 1997; Mueller and others 1998; Thomas and others 1998a)
D. pteronyssinus *D. farinae*	3	Der p 3 Der f 3	17-30	Trypsin	(O'Hehir and others 1993; Thomas and others 1998a)
D. pteronyssinus *D. farinae*	4	Der p 4 Dr f 4	56-60	Amylase	(O'Hehir and others 1993; Thomas and others 1998a)
D. pteronyssinus *D. farinae*	5	Der p 5 Der f 5	15	?	(O'Hehir and others 1993; Thomas and others 1998a)
D. pteronyssinus *D. farinae*	6	Der p 6 Der f 6	25	Chymotrypsin	(O'Hehir and others 1993; Thomas and others 1998a)
D. pteronyssinus *D. farinae*	7	Der p 7 Der f 7	22-28	?	(O'Hehir and others 1993; Thomas and others 1998a)
D. pteronyssinus	8	Der p 8	?	Glutathione-S-transferase	(Thomas and others 1998a)
D. pteronyssinus	9	Der p 9	?	Serine protease	(Thomas and others 1998a)
D. pteronyssinus *D. farinae*	10	Der p 10 Der f 10 Mag 44	60-65	Tropomyosin	(Thomas and others 1998a) (Reese and others 1999) (Fujii and others 1997)
D. pteronyssinus *D. farinae*	11	Der p 11 Der f 11	92-98	Paramyosin	(Thomas and Smith 1999) (Tsai and others 1999)
D. pteronyssinus *D. farinae*	12	Der p 12 Der f 12	14	?	(Thomas and Smith 1999)
D. pteronyssinus *D. farinae*	13	Der p 13 Der f 13	15	Fatty acid binding protein	(Thomas and Smith 1999)
D. farinae	15	Der f 15 b	97-109 95-101	Chitinase	(McCall and others 2000) (Le Mao and others 1998)
D. farinae	?	a	80-83	?	(Le Mao and others 1998)
D. farinae	? 14 ?	Mag 1 Mag 3 / Der f 14 Mag 29	39 177 70	Apolipophorin Apolipophorin Heat shock protein	(Fujii and others 1997; Epton and others 2001) (Kawamoto and others 2000)
D. farinae	16	Der f16	55	Gelsolin	(Kawamoto and others 2002)

1.5.5.2 *Allergens relevant to dogs*

Dermatophagoides species are equally important in canine atopic dermatitis (Sture and others 1995), although the spectrum of *Dermatophagoides* allergens relevant to dogs is less clear. Most atopic dogs in one study had positive skin tests and serum specific IgGd to crude *D. farinae* and *D. pteronyssinus* extracts, but not to purified group 1 and 2 allergens (Noli and others 1996). IgGd binding proteins with molecular weights of 65-70kD and 90kD were identified on western blots, but no binding to group 1 or 2 allergens was seen (Noli and others 1996). Another study used recombinant canine FcεRIα to detect IgE binding proteins on western blots of *D. farinae*. Eighty-five per cent of the atopic sera recognised 60, 98 and 109kD allergens, but 69% of the atopic sera also recognised Der f 1 (McCall and others 1999). Homology of the 98kD protein with published sequences suggests it is a chitinase. It has recently been cloned, expressed and named Der f 15 (McCall and others 2000). An independent investigation using FcεRIα found that the majority of atopic sera also recognised 98 and 109kD allergens in *D. pteronyssinus* (Shaw 2000). A study using monoclonal anti-canine IgE in enzyme linked immunosorbent assays and dot blots, however, reported that 50% of atopic sera recognised Der p 1 and 2, and Der f 1 and 2 (Masuda and others 1999).

1.5.5.3 *Homology and polymorphisms of* D. pteronyssinus *and* D. farinae *allergens*

There is generally 80-90% homology between *D. pteronyssinus* and *D. farinae* allergens (Mueller and others 1998; Smith and others 1999). However, spontaneous polymorphisms of group 1 and group 2 allergens are recognised (O'Brien and others

1995). Regional polymorphisms could account for the differences in IgE binding capacity between allergen extracts (van Neerven and others 1996; Thomas and others 1998b). Interestingly, the most polymorphic regions of Der p 1 and Der p 2 coincide with the immunodominant peptide sequences (O'Brien and others 1995). These polymorphic sequences could permit sensitisation to group 1 and 2 allergens across a range of HLA haplotypes. In contrast, more stringent HLA restriction for the highly conserved group 5 allergens might explain why sensitisation to these allergens is less prevalent (O'Brien and others 1995).

1.5.6 Allergenic properties of *Dermatophagoides* allergens

1.5.6.1 Structural properties

Only a minority of proteins induce an allergic response, which suggests that they possess intrinsic allergenic properties (Comoy and others 1999). Despite this, there are few common patterns. Allergenic proteins tend to be stable in the environment and *in vivo* (Bufe 1998; Huby and others 2000), which enhances allergen exposure, uptake and processing. Disulphide bonds appear to be important in stabilising the three dimensional structure of IgE epitopes, as their disruption abrogates allergenicity (Huby and others 2000). Many allergens, including Der f 15, the 98kD chitinase important in canine atopic dermatitis, are also glycosylated. Glycosylation increases IgE binding affinity, B-cell recognition, and antigen uptake and presentation by APCs (Huby and others 2000). Allergens may also directly influence the immune system. Group 11 allergens, for example, can inhibit complement activation (Tsai and others 1999; Tsai and others 2000). Group 1 and 2 allergens can also induce expression of MHCII and CD80 on keratinocytes, rendering them capable of presenting antigens to acti-

vated T-cells *in vitro* (Wakem and others 2000). Protein allergens can also inhibit IL-12 by stimulating IL-10 production (Bellinghausen and others 1999).

1.5.6.2 Enzymatic activity

Much interest has focused on the biological activity of allergens. Most *Dermatophagoides* allergens are enzymes that retain protease activity in the environment and *in vivo* (Shakib and Furmonaviciene 2000). Group 1 allergens are cysteine proteases with stable proteolytic activity *in vivo* and *in vitro*, even when complexed with specific antibody (Schulz and others 1999). Der p 1 cleaves occludin/claudin family tight junction proteins and E-cadherin, resulting in disruption of epithelial cell architecture and detachment of epithelial cell monolayers *in vitro* (Wan and others 1999; Wan and others 2000). This could impair barrier defences, increasing allergen uptake and microbial colonisation. Der p 1 also inactivates α1-antitrypsin, enhancing exogenous and endogenous protease activity (Bufe 1998; John and others 2000). Furthermore, Der p 1 specifically cleaves surface CD23 and CD25, the IL-2 receptor. Decreased negative feedback from surface CD23 and increased soluble CD23 enhances IgE production by B-cells (Bufe 1998; Schulz and others 1999; Tsicopoulos and Joseph 2000). Loss of CD23 also inhibits monocyte and macrophage activation (Bufe 1998; Schulz and others 1999). Cleavage of CD25 strongly inhibits proliferation of activated T-cells and IFNγ production *in vitro* (Bufe 1998; Schulz and others 1999). Der p 1 can also activate mast cells in an IgE independent manner (John and others 2000). Blocking the proteolytic activity of Der p 1 in mouse models of asthma resulted in decreased IgE production and ameliorated airway pathology (John and others 2000; Shakib and Gough 2000).

1.6 Immunoglobulins in atopic dermatitis

1.6.1 IgE

IgE plays a pivotal role, both at the effector level through binding to FcεRI on mast cells and basophils and in enhancing allergen presentation *via* binding to FcεRI and CD23 on Langerhans' cells, dendritic cells, monocytes and B-cells (Leung 1998). On subsequent exposure to allergen, cross-linking of surface IgE triggers synthesis and release of inflammatory mediators, immunoglobulins, receptor expression, allergen uptake, processing and presentation (Maggi 1998; Okudaira and Mori 1998). Cross-linking of surface IgE by allergen, furthermore, induces FcεRI and IgE synthesis (Helm and others 1998).

Total serum IgE is significantly correlated with atopy in humans, particularly infants (Leung 1999), but not in dogs, where a high level of parasitism may mask differences (Hill and others 1995; Racine and others 1999). Serum IgE levels in dogs rise up to four years of age and then plateau, which coincides with the peak age of onset for atopic dermatitis (Racine and others 1999). Female dogs have higher IgE levels (Racine and others 1999), but no consistent female bias in atopic dermatitis has been found (Scott and others 2001a).

Levels of allergen specific IgE are frequently elevated in human and canine atopic dermatitis and are considered a hallmark of the disease (Leung 2000; Halliwell and DeBoer 2001). Furthermore, serological or skin tests are routinely employed to identify allergens for avoidance or specific immunotherapy (Pachlopnik and others 1999; Scott and others 2001a). However, allergen specific serum IgE is also present in healthy dogs and humans, suggesting that heterogeneous IgE antibodies with variable receptor binding affinities exist (Halliwell and others 1998; Griot-Wenk and others

1999; Marone and others 1999). Recent findings suggest that two distinct subtypes of canine IgE exist (Peng and others 1993). The presence of positive skin tests in some healthy individuals though (Halliwell and others 1998; Lian and Halliwell 1998; Beltrani 1999b), suggest that other tolerogenic mechanisms are also present.

1.6.2 IgG

It is likely that most, if not all, individuals are exposed and respond to allergens as specific IgG is found in both atopic and healthy humans (Upham and others 1995) and dogs (Day and others 1996). The role of IgG in atopic dermatitis is unclear. Allergen-specific and anti-idiotype IgG antibodies may block IgE activity in healthy individuals (Griot-Wenk and others 1999). Eighty per cent of human atopic patients have IgG/IgE complexes (Marone and others 1999), although it is unclear whether these are biologically irrelevant, block the functional activity of IgE or induce pro-inflammatory mediator release.

Allergen-specific IgG levels correlate with IgE levels in canine atopic dermatitis. Elevated IgE titres to dust and mite allergens were detected more frequently than IgG titres, although the reverse was true for insect mix and mould allergens (Day and others 1996). Responses to mite allergens were dominated by the IgG4 subclass, whereas IgG1 antibodies dominated responses to Timothy grass (*Phleum pratense*). This difference may be due to the biological nature of the allergens, route of exposure or antigen processing and presentation. However, it is unclear if this has any pathological significance.

It has also been suggested that IgGd subclass antibodies are also capable of inducing mast cell degranulation in dogs and that allergen-specific IgGd antibodies are largely

specific to canine atopic dermatitis (Willemse and others 1985). However, monoclonal antibody specific for IgGd did not bind to any of the canine IgG subclasses 1-4 in one study (Mazza and others 1993). A recent study of allergen specific IgGd and IgE in both atopic and healthy dogs found that levels of IgE correlated better with the diagnosis of canine atopic dermatitis (Lian and Halliwell 1998). These findings, plus a lack of reverse cutaneous anaphylaxis and basophil histamine release prompted a recent review to conclude that there is only circumstantial evidence that IgG is involved in the pathogenesis of canine atopic dermatitis (Halliwell and DeBoer 2001).

1.7 Antigen presenting cells and allergen presentation in atopic dermatitis

Immunoglobulin synthesis by B-cells is dependent on interaction with a $CD4^+$ T-helper cell (T_H cell) activated by an antigen-presenting cell (APC) (Huston 1997). Non-professional APCs (including macrophages, endothelial cells, keratinocytes and B-cells) are incapable of stimulating naïve cells, and are more important in presenting antigen to activated T-cells in peripheral tissues (Huston 1997; Kapsenberg and others 1998; Robert and Kupper 1999). In particular, B-cells bearing IgE bound to CD23 are very effective at presenting allergen to T-cells (Mudde and others 1996). In contrast, only professional APCs, such as epidermal Langerhans' cells and dermal dendritic cells, can activate naïve T-cells in draining lymph nodes (Mudde and others 1996; Kapsenberg and others 1998).

In peripheral tissues, APCs are geared towards antigen uptake and processing. Langerhans' cells in both human and canine atopic dermatitis express FcεRI (Bieber 1997; Olivry and others 1999a). IgE binding enhances allergen uptake 100-1000 fold

(Bieber 1997; Cox and others 1998). The phagocytosed allergens are then exposed to lysosomal acid proteases and cathepsins. By a process of low pH, reduction, oxidation, unfolding, and proteolysis, these degrade the allergens into 10-15 amino acid peptides that include the T-cell receptor (TCR) epitopes (Carrasco-Marín and others 1998). The peptides are assembled with MHCII molecules and are transported to the cell surface for presentation to $CD4^+$ T_H cells (Kalish and Askenase 1999).

On exposure to allergen and inflammatory cytokines, Langerhans' cells and dendritic cells down regulate surface adhesion molecules and migrate to the local lymph node. Here they lose the ability to process antigens, but have enhanced antigen presenting capacity expressing MHCII/peptide complexes and co-stimulatory molecules (Bieber 1997; Kapsenberg and others 1998; Kimber and others 1998).

1.8 The role of T-cells in atopic dermatitis

1.8.1 Recruitment and activation of $CD4^+$ cells

Naïve ($CD45RA^+$ in humans) $CD4^+$ T_H cells express ligands for L-selectin on peripheral lymph node high endothelial venules, where they come into contact with APCs (Huston 1997). Allergen/MHCII complexes are recognised by specific TCR/CD4 complexes on T_H cells. Ligation of CD80/86 with CD28, CD40/CD40L and cytokine expression provide the accessory signals necessary for differentiation into activated ($CD45RO^+$ in humans) $CD4^+$ T_H cells (Delves and Roitt 2000). Activated $CD4^+$ T_H cells then migrate into the peripheral tissues. T-cells in atopic dermatitis express CLA, a ligand for E-selectin expressed on dermal blood vessels, as well as ligands for other selectins and chemokines in inflamed skin (Leung 1998; Tu and others 1999).

1.8.2 T_{H1} and T_{H2} Cells

Subsets of T_H cells were first described in mice and rats (Arthur and Mason 1986; Mosmann and Coffman 1987), when clones supporting cell mediated immune responses (T_{H1}) were distinguished from clones providing B-cell help (T_{H2}). Since then T_{H1} and T_{H2}-like cells have been found in humans and other species (Romagnani 1991; Maggi 1998).

1.8.2.1 T_{H1} responses

T_{H1}-type cytokines (Table 1.2) mediate delayed (type IV) hypersensitivity reactions, activate macrophages, cell mediated immune responses, production of opsonising and complement fixing IgG and IgM, and antibody dependent cell-mediated cytotoxicity (Abbas and others 1996). Their primary role is in clearing infectious agents, particularly intracellular organisms, tumours, endogenous and insoluble antigens (Abbas and others 1996; Kalish and Askenase 1999). However, T_{H1} responses can damage host tissues, especially in chronic inflammatory and immune-mediated diseases (Leung 1998; Maggi 1998).

Table 1.2 – T_{H1} and T_{H2} type cytokines

T_{H1} cytokines	**T_{H2} cytokines**
IL-2	IL-4
IL-12	IL-5
IL-18	IL-6
IFNγ	IL-10
TNFα	IL-13

IL-2 promotes lymphocyte proliferation, survival and differentiation (Helfand and others 1992). Autocrine IL-2 secretion drives expansion of T_{H1} clones (Maggi 1998).

IL-12 drives T_{H1} differentiation of naïve T_H cells (Maggi 1998). Functional IL-12 is a heterodimer, consisting of a p35 and p40 subunit (McDyer and others 1998). p35 dimers are biologically inactive, whilst the p40 dimer blocks IL-12 activity (Piccotti and others 1996). IL-12 is produced primarily by APCs and macrophages, but also by B-cells, neutrophils, keratinocytes and eosinophils (McDyer and others 1998). Stimuli for IL-12 synthesis and release include specific antigens, intracellular pathogens and IFNγ (McDyer and others 1998; Kimber and others 2000). In contrast, IL-4 and IL-10 inhibit IL-12 production (McDyer and others 1998; Camporota and Holloway 1999; Kimber and others 2000).

IL-18 supports T_{H1} differentiation and cell mediated inflammation, and inhibits IgE production with IL-12 (Kimber and others 1998; McDyer and others 1998; Dinarello 1999), but supports IL-4 mediated activity in the absence of IL-12 (Yoshimoto and others 2000).

IFNγ up regulates expression of MHCII, adhesion molecules, pro-inflammatory cytokines and chemokines by keratinocytes and dermal cells. These molecules are important in attracting, retaining and activating T-cells, macrophages and other inflammatory cells (Maggi 1998; Pastore and others 1998). IFNγ also inhibits mast cell FcεRI expression, cytokine release (Tachimoto and others 2000) and encourages apoptosis (Rossi and others 1998).

TNFα is produced by T_{H1} cells, macrophages, mast cells and basophils (Maggi 1998; Kobayashi and others 2000). It is strongly pro-inflammatory and levels correlate with the severity of clinical signs in allergic respiratory disease (Kobayashi and others

2000). TNFα also increases the activity of dendritic cells (Skok and others 1999) but inhibits mast cells, FcεRI expression and cytokine release (Rossi and others 1998).

1.8.2.2 T_{H2} responses

T_{H2} cytokines (Table 1.2), in contrast, promote mast cell, eosinophil and B-cell growth and development, production of IgA, IgE, and non-opsonising and complement fixing IgG and IgM (Abbas and others 1996; Till and others 1997; de Vries 1998). They also induce adhesion molecules on endothelial cells (Ramírez and others 1998; Sherman and others 1999). T_{H2} responses are less effective against infectious agents, but are important in responses to parasites and soluble antigens (Abbas and others 1996). They are also less damaging to the host under normal circumstances, and may help to regulate T_{H1} responses (Leung 1998; Maggi 1998).

IL-4 and the closely related cytokine IL-13 induce IgE production, antigen presentation, expression of MHCII, CD23 and adhesion molecules, and eosinophil chemotaxis and activation (Monticelli and others 1998; Corry 1999; Luttmann and others 1999). They inhibit the production of opsonising and complement fixing IgG and IgM, and macrophage activation (Fiorentino and others 1989; Abbas and others 1996; Ryan 1997). In humans, IL-4 induces FcεRI expression, whereas it is inhibitory in mice (Bieber 1997; Ryan and others 1998). IL-4 also increases mast cell proliferation and cytokine release (Rossi and others 1998; Yamaguchi and others 1999; Tachimoto and others 2000).

T_{H2} differentiation is dependent on IL-4. Early sources of IL-4 could include T_{H2} cells, NK cells, mast cells, eosinophils, basophils and APCs (Bottomly 1988; Ryan 1997; de Vries 1998; Maggi 1998; Haas and others 1999). It possible that IL-4 is

produced by cells of the innate immune system, such as basophils and mast cells in response to allergen contact (Haas and others 1999). However, this does suppose prior T_{H2} differentiation and sensitisation with allergen-specific IgE. Other studies have shown that naïve $CD4^+$ cells can produce IL-4 on priming, suggesting that T_{H2} differentiation is determined by autocrine secretion of IL-4 (Bullens and others 1999). Subsequent expansion of T_{H2} clones depends on autocrine IL-4, which enhances responses to IL-2 (van Reijsen and others 1992).

IL-13 is produced by T_{H2} cells, B-cells, dendritic cells, mast cells, basophils, NK cells and keratinocytes (de Vries 1998; Corry 1999). It probably plays little role in differentiation of $CD4^+$ cells, which lack IL-13 receptors (de Vries 1998; Corry 1999). However, epicutaneous sensitisation can still induce T_{H2} differentiation in IL-4 knockout mice, implying that IL-13 might provide an IL-4 independent pathway for T_{H2} differentiation in the skin (Herrick and others 2000). IL-13 also synergises with SCF to promote mast cell growth, differentiation and activity (de Vries 1998). In humans, IL-13 is produced by T_{H1} cells in late phase inflammation, which may allow cross-regulation of T_{H1} and T_{H2} responses (de Vries 1998). Recombinant canine IL-13 also stimulates IgE production by PBMCs isolated from dogs sensitised to flea allergens (Tang and others 2001), suggesting that IL-13 is also an important mediator of IgE production in canine allergic disease.

IL-5 recruits and activates eosinophils, which is crucial in late phase inflammatory responses (Okudaira and Mori 1998; O'Byrne and Wood 1999). IL-5 also supports IgE synthesis by IL-4 stimulated B-cells and enhances basophil histamine release (Lalani and others 1999). IL-5 secretion by T_{H2} cells, mast cells and eosinophils is stimulated by IL-2, histamine, PGE_2 and IgE. Secretion is inhibited by transforming

growth factor beta (TGFβ), IL-10, IFNγ and IL-12 (Lalani and others 1999; O'Byrne and Wood 1999).

Many cells, including APCs, macrophages, mast cells, T_{H2} cells, fibroblasts, endothelial cells and keratinocytes, produce IL-6. It primarily supports B-cell development, IgG and IgE production. However, it also induces IL-4 and antagonises IL-12, and may therefore be an early T_{H2} differentiating signal (Kirman and Le Gros 1998; Skok and others 1999).

1.8.2.3 T_{H1} and T_{H2} cytokine profiles

Whilst murine T_H cells have a restricted cytokine profile, in human cells this is much less so. Individual cells often secrete a complex set of cytokines including IL-2, IL-4, IL-5, IL-10, IFNγ and TGFβ (Abbas and others 1996; Neumann and others 1996; Umetsu and DeKruyff 1997; Grewe and others 1998). However, T_{H1} (high IFNγ) and T_{H2} (high IL-4) polarised responses can still be distinguished (Abbas and others 1996; Borish and Rosenwasser 1997; Maggi 1998).

1.8.2.4 Antagonism of T_{H1} and T_{H2} responses

T_{H1} and T_{H2} differentiation in mice is mutually antagonistic (Fiorentino and others 1989; Abbas and others 1996). Furthermore, human patients with multiple sclerosis (a T_{H1} mediated disease) have a low risk for atopy (Umetsu and DeKruyff 1997), whereas atopic patients have a low incidence of allergic contact dermatitis (also T_{H1} mediated) (Cooper 1994). Depressed cutaneous cell mediated immunity has also been reported in canine atopic dermatitis (Nimmo Wilkie and others 1991). Despite this, other studies have shown that human atopic patients with T_{H2} skewed responses

to house dust mite allergens still mount a normal T_{H1} response to PPD antigen (Nurse and others 2000). T_{H1} and T_{H2} responses to independent antigens, furthermore, can co-exist in healthy individuals (Ismail and Bretscher 1999), implying that normally there is a T_{H1}/T_{H2} balance. Pathological imbalances, however, could lead to an inappropriate T_{H1} (e.g. immune-mediated diseases) or T_{H2} (e.g. hypersensitivity diseases) responses (Umetsu and DeKruyff 1997).

1.8.2.5 The role of APCs in T_{H1} and T_{H2} differentiation

T_H cell differentiation is directed by signals in the local microenvironment during antigen presentation and activation. The expression of cytokines and co-stimulatory molecules by APCs is determined by the dose, type and route of allergen exposure, as well as other inherent and environmental factors (Kapsenberg and others 1998; Rissoan and others 1999; Bottomly 1999).

In mice, IL-12 and IFNγ support maturation of type 1 dendritic cells (DC1) that induce T_{H1} responses, whilst PGE_2 promotes DC2 maturation and T_{H2} responses (Kapsenberg and others 1998). Elevated PGE_2 levels due to increased phosphodiesterase activity have been observed in PBMCs from atopic humans (Hanifin 1996) and APCs from atopic individuals produce less IL-12 than those from healthy individuals (De Becker and others 1998; Stumbles and others 1998; van Neerven and others 1998).

The potency of allergen/TCR/MHCII ligation is also important. Low potency TCR signals drive T_{H2} differentiation, whereas high potency signals favour T_{H1} (Maggi 1998; Grakoui and others 1999; Leitenberg and Bottomly 1999). Potency is determined by affinity and density. It is thought that low density and/or affinity stimulate

low cytokine levels permissive of IL-4 mediated T_{H2} differentiation. At higher densities or affinity IL-12 production overcomes IL-4 inhibition and drives T_{H1} expansion (Grakoui and others 1999), although very low affinity allergens are density independent (Röcken and others 1992).

It is also likely that the proteolytic enzyme repertoire of APCs influences T-cell differentiation (Bufe 1998). Inhibitors of cathepsin B, a lysosomal protease involved in antigen processing, switched T-cell differentiation from T_{H2} to T_{H1} in a mouse model of Leishmaniasis (Maekawa and others 1998).

Neuropeptides such as somatostatin, substance P and vasoactive intestinal polypeptide can also influence allergen presentation, T_H cell activation, cytokine secretion and expression of adhesion molecules (Levite 1998). This may allow neuroendocrine networks to fine tune immune responses.

1.8.3 The T_{H1}/T_{H2} hypothesis in atopic dermatitis

1.8.3.1 T_{H2} cytokines in human atopic dermatitis

Numerous studies have associated human atopic dermatitis with T_{H2} polarisation. Non-lesional skin and acute lesions exhibit increased transcription of mRNA for IL-4 and IL-13 compared to healthy skin (Leung 2000). Furthermore, successful immunotherapy is associated with a switch from a T_{H2} dominant pattern to a T_{H1} dominant pattern (Renz 1995; Smith and Sly 1998).

Several studies have isolated allergen specific T_{H2} clones from PBMCs and the skin of atopic patients (van der Heijden and others 1991; Cooper 1994; Renz 1995; Neumann and others 1996; Ochi and others 1996; Poulsen and others 1996; Ferry and others 1997; Koning and others 1997a; Koning and others 1997b; Maggi 1998).

These clones over-express the T_{H2} cytokines IL-4 and IL-5 and under-express the T_{H1} cytokines IFNγ and IL-2 compared to T-cell clones from healthy individuals. In contrast, stimulation of PBMCs with non-allergenic antigens expands non-polarised or T_{H1} clones (Romagnani 1998).

In some studies, however, whilst T_{H2} cytokine production was still elevated, T_{H1} cytokine expression was similar to that in healthy individuals (Till and others 1997; Lonati and others 1999). In others, T_{H1} cytokine production was reduced with no effect on T_{H2} cytokines (Poulsen and others 1996). These results probably reflect differences in experimental design, methodology and kinetics of T_{H1} and T_{H2} cytokine production, as the net effect in each case was T_{H2} polarisation.

Several studies suggest that allergen specific T_{H2} polarisation is important in the pathogenesis of atopic dermatitis. The frequency of T_{H2} clones isolated from the skin usually exceeds that from PBMCs (Grewe and others 1998), suggesting there is either selective recruitment of T_{H2} cells or local T_{H2} differentiation in the skin. Patch testing, furthermore, generates allergen specific T_{H2} clones within 24 hours (van der Heijden and others 1991; van Reijsen and others 1992; Werfel and others 1996) and T_{H2} polarisation is most prominent in patients with high IgE levels (Ochi and others 1996; Rodríguez and others 1998; Sato and others 1998; Kimura and others 2000).

1.8.3.2 T_{H1} cytokines in human atopic dermatitis

T_{H1}-type cytokines are also expressed in atopic patients. Whereas IL-4^+ cells predominate in acute lesions, IFNγ^+ cells dominate chronic lesions (Werfel and others 1996). Furthermore, IFNγ levels correlate with the clinical severity and are reduced after successful immunotherapy (Werfel and others 1996; Grewe and others 1998).

Patch testing with house dust mite allergens demonstrated that IL-4$^+$/IFNγ$^-$ cells predominate up to 24 hours and then decline. Peak levels of IL-4$^+$/IFNγ$^+$ and IL-4$^-$/IFNγ$^+$ cells were attained at 24 and 48 hours respectively and then maintained (Thepen and others 1996; Grewe and others 1998; Junghans and others 1998). TNFα is also over expressed in chronic lesional compared to healthy skin (Junghans and others 1998).

1.8.3.2 T_{H1} and T_{H2} cytokine expression in mouse models of atopic dermatitis

Acute inflammation was associated with IL-4 and IL-5 in two mouse models of atopic dermatitis induced by cutaneous application of ovalbumin. Chronic inflammation and skin thickening, in contrast, was driven by IFNγ (Spergel and others 1999; Vestergaard and others 1999). IFNγ knockout mice, furthermore, exhibit a marked reduction in skin thickening (Leung 2000).

Other evidence suggests that T_{H1} cytokines potentiate established patterns of T_{H2} inflammation and *vice versa*. Administration of IL-4 worsened experimental allergic encephalomyelitis (a T_{H1} model of multiple sclerosis) and supported proliferation of activated T_{H1} cells, whilst IFNγ worsens T_{H2} induced bronchial hyper-reactivity (Hansen and others 1999; Oriss and others 1999).

1.8.3.4 Sequential cytokine expression in human atopic dermatitis

These findings suggest a sequential pattern of T_H cell activation and cytokine production. Naïve T_H cells differentiate into T_{H2} cells under the influence of allergen presentation by IgE$^+$ APCs in the lymph nodes and possibly skin. T_{H2} cells initiate IgE production by B-cells, which further primes mast cells and APCs. On contact with aller-

gen, IgE^+ mast cells degranulate, triggering immediate and late phase inflammatory reactions, which recruit eosinophils and mononuclear cells. Subsequent IL-12 and IFNγ production initiates differentiation of allergen specific (Werfel and others 1996) or non-specific (Cooper 1994) T_{H1} cells and chronic cell mediated inflammation (Borish and Rosenwasser 1997; Grewe and others 1998), without affecting IgE production. Human T_{H2} cells are resistant to activation-induced apoptosis (Carbonari and others 2000), which may favour the development of stable T_{H2} populations.

IL-12 expression precedes that of IFNγ and coincides with eosinophil and macrophage infiltration (Grewe and others 1998). These cells are recruited by IL-4 and IL-5 and may provide signals for the switch to T_{H1} mediated chronic inflammation (Grewe and others 1998). Histamine also induces IL-18 expression, which promotes T_{H1} differentiation and IFNγ production in human PBMCs (Kohka and others 2000).

T_{H1}-type cell recruitment could also be due to a variety of non-specific mechanisms. Keratinocytes rapidly release TNFα, IFNγ, IL-1 and granulocyte-monocyte colony stimulating factor (GM-CSF) in response to trauma (Leung 2000). Levels of GM-CSF are increased in atopic compared to healthy keratinocytes (Leung 2000), which would enhance the survival of mononuclear cells and eosinophils.

1.8.3.5 T_{H1} and T_{H2} cytokine expression in canine atopic dermatitis

Studies of the cytokine profile in canine atopic dermatitis have been limited. One study (Olivry and others 1999b) demonstrated a T_{H1}-like pattern in 25% of healthy skin samples, a T_{H2}-like pattern in 25% of atopic samples, and a mixed cytokine pattern in the rest. Another detected IL-4 in atopic lesional skin (Sinke and others 1998) and patch test sites (Rutten and others 2001). However, one study failed to detect IL-

4 (Maeda and others 2002) and it remains to be proven that canine atopic dermatitis is associated with T_{H1}/T_{H2} polarisation similar to that described in humans.

1.8.4 $CD8^+$ cells in atopic dermatitis

IFNγ rich (T_{C1}) and IL-4 rich (T_{C2}) $CD8^+$ subsets have also been identified in humans (Kemeny 1998; Maggi 1998; Akdis and others 1999b; Kalish and Askenase 1999). However, their role in atopic dermatitis is unclear. T_{C1} cells could mediate chronic inflammation and/or limit T_{H2} mediated IgE production (Renz 1995; Kemeny 1998). In contrast, T_{C2} cells could support T_{H2} differentiation and IgE production during chronic inflammation (Kemeny 1998). $CD8^+$ cells are MHCI restricted (Huston 1997), which implies that these cells are not allergen specific (Akdis and others 1999b), or if allergen specific (O'Brien and others 2000), that exogenous allergens can also be presented with MHCI molecules (Kemeny 1998), blurring the distinction between type I and type IV hypersensitivity.

1.9 Mast cells in atopic dermatitis

Mast cells are sentinels at interfaces with the external environment, such as the gastro-intestinal tract, respiratory tract and skin. They play a central role in normal tissue metabolism and turnover, bacterial infections, sterile inflammation, parasite infestations, wound healing and remodelling (Welle 1997; Galli and others 1999). They trigger immediate phase innate responses, but also regulate long term inflammatory reactions by linking the innate and acquired immune systems, vascular and neuroendocrine systems (Church and Levi-Schaffer 1997; Welle 1997; Brody and Metcalfe 1998; Galli and others 1999).

1.9.1 Mast cell development

$CD34^+$ precursors of haemapoietic origin migrate out of the circulation and mature in peripheral tissues under the influence of IL-3, IL-4, IL-9 and IL-10. However, ligation of the *c-Kit* receptor on mast cells by SCF, is crucial in mast cell recruitment, development and survival (Church and Levi-Schaffer 1997; Welle 1997; Galli and others 1999; Williams and Galli 2000). SCF or *c-Kit* gene knockout mice are mast cell deficient and mutations in *c-Kit* and SCF may be important in mast cell tumours (London and others 1996; Yongsheng and others 1999).

SCF is produced by fibroblasts, keratinocytes, endothelial cells, epithelial cells, Langerhans' cells, mast cells and lymphocytes (Church and Levi-Schaffer 1997). In humans, there are both soluble and membrane bound forms, which may be important direct cell-cell interactions (de Paulis and others 1999).

c-Kit is expressed by mast cells, melanocytes, cord blood mononuclear cells, foetal hepatocytes and bone marrow progenitor cells (Church and Levi-Schaffer 1997; de Paulis and others 1999). Its presence on intraepithelial lymphocytes may be important in SCF mediated regulation of gastro-intestinal tract lymphocytes (Wang and others 2000a). Inhibition of *c-Kit* expression by SCF, IL-4 and IL-10 may be a feedback mechanism to limit mast cell activity (Mirmonsef and others 1999).

c-Kit and SCF are expressed by canine mast cells and stromal cells respectively (London and others 1996; Kapsenberg and others 1998). Structural similarities suggest that regulation of mast cell development is similar to that in humans and rodents, although studies of functional activity in dogs are limited.

1.9.2 Cutaneous mast cell phenotypes in humans and dogs

Two types of mast cells have been identified in humans - tryptase (MC_T) and tryptase-chymase (MC_{TC}) (Church and Levi-Schaffer 1997; Welle 1997). Mixed populations occur in most tissues, although MC_T are mostly mucosal and MC_{TC} are predominantly located in connective tissue (Church and Levi-Schaffer 1997). Whilst both express FcεRI, MC_T are thought more important in allergic and parasitic reactions, whilst MC_{TC} regulate angiogenesis and tissue repair (Church and Levi-Schaffer 1997; Welle 1997). IL-4 is expressed by both MC_{TC} and MC_T, but IL-5 and IL-6 are exclusive to MC_T (Welle 1997).

MC_T and MC_{TC}-like cells have also been described in canine skin (Caughey 1990; Welle and others 1999). Selective degranulation of MC_T-like cells in atopic skin (Welle and others 1999) suggests that these cells play a specific role in canine atopic dermatitis.

1.9.3 FcεRI expression and IgE binding

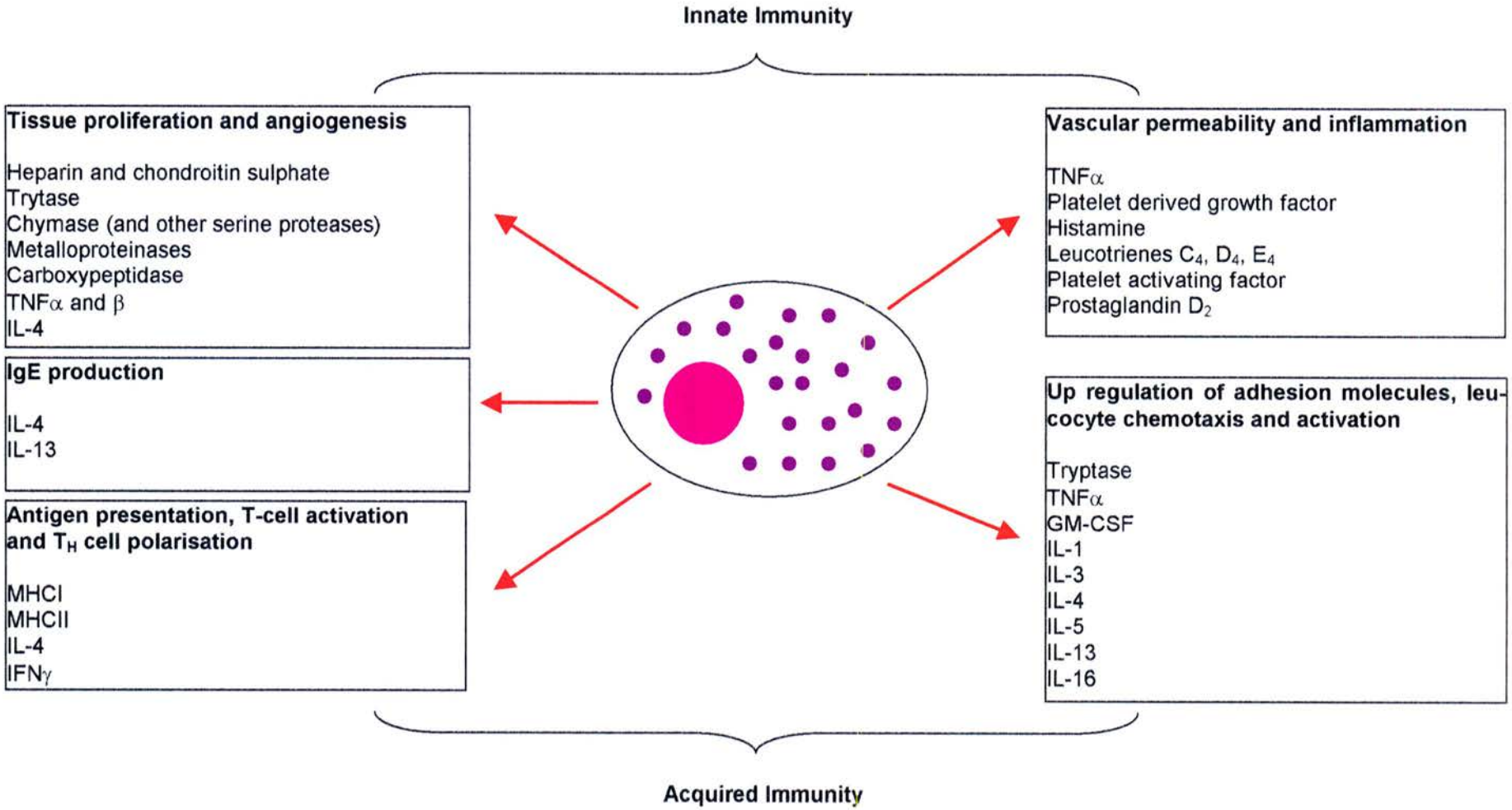
FcεRI is expressed by mast cells early in differentiation (Williams and Galli 2000) and is dependent on IL-4 (Chen and others 1999; Tachimoto and others 2000). IgE binding to FcεRI in the skin, ocular and pulmonary mucosa is stable, outlasting detectable serum levels of IgE in experimentally sensitised dogs (Zeman and others 2002). IgE also up regulates FcεRI expression on human PBMCs (Yamaguchi and others 1999) and canine mast cells (Brazís and others 2002) *in vitro*.

1.9.4 Mast cell degranulation

There is strong evidence of IgE dependent mast cell responses to specific allergens in canine and human atopic skin (Leung 2000; Hill and Olivry 2001). Cross-linking of surface IgE by allergens triggers the release of a variety of preformed and stored mediators that initiate immediate phase inflammatory responses, inflammatory cell infiltrates and late phase reactions (Brody and Metcalfe 1998; Williams and Galli 2000) (Figure 1.4). The immediate phase inflammatory response is largely mediated by the release of preformed vasoactive mediators, such as histamine, kallikrein and heparin. Late phase reactions are mediated by newly synthesised inflammatory eicosanoids, chemokines and cytokines that recruit and activate eosinophils, mononuclear cells and lymphocytes (Maggi 1998; Okudaira and Mori 1998; Leung 2000).

Immediate phase reactions are dependent on mast cell degranulation and are abrogated in mast cell deficient mice. Late phase responses involving T-cells, macrophages and eosinophils can be mast cell independent, but mast cell activation is necessary for the co-ordinated expression of immediate and late phase inflammation (Williams and Galli 2000). Late phase responses in atopic dogs can be induced by either specific allergen or anti-canine IgE (Olivry and others 2001), suggesting that they are also initiated by IgE mediated mast cell degranulation. Mast cell degranulation tends to be abrupt and complete in acute inflammation, whereas chronic inflammation is associated with mast cell hyperplasia and gradual degranulation (Welle 1997).

Figure 1.4 – The functional effects of mast cell mediators released on activation (Church and Levi-Schaffer 1997; Welle 1997; Brody and Metcalfe 1998; Williams and Galli 2000)



1.9.5 The role of mast cells in atopic dermatitis

Allergen induced activation of mast cells is widely believed to initiate inflammatory reactions in atopic skin (Welle 1997). However, it is unclear whether they actively participate in the development of atopic dermatitis or whether they are simply activated in an inappropriate context (Brody and Metcalfe 1998).

Increased mast cell density and histamine concentrations have been detected in human (Irani and others 1989) and canine atopic dermatitis (Nimmo Wilkie and others 1990). In healthy dogs, mast cell numbers are enhanced in the pinnae, ventral and interdigital skin (Auxilia and Hill 2000), which are all predilection sites for atopic dermatitis. Another study demonstrated that mast cells numbers in the dorsal and ventral body are significantly increased in atopic compared to healthy skin (Nimmo Wilkie and others 1990). Furthermore, mast cells isolated from atopic canine skin contained more histamine and were more reactive to concanavalin A than mast cells from healthy skin (de Mora and others 1996). However, the role of histamine in atopic dermatitis is uncertain. Antihistamines are only of limited benefit in humans (Beltrani 1999b) and dogs (Scott and others 2001a), and other studies in humans have failed to show an increase in plasma or cutaneous histamine (Beltrani 1999b). In addition, *in vitro* sensitised mast cells from atopic and normal canine skin had a similar response to anti-IgE, suggesting *in vivo* sensitisation was more important than inherent activity (Brazís and others 1998).

Bone marrow derived mast cells (BMMC) in mice express MHCI and II with CD80/CD86 and can present allergens to activated $CD4^+$ cells *in vitro*. The *in vivo* significance of this, however, is unclear (Rossi and others 1998; Mekori and Met-

calfe 1999). Murine BMMC can also induce T-cell independent B-cell activation and IgE production *in vitro* (Rossi and others 1998).

Mast cells could also contribute to the IL-4 dominated cytokine milieu in atopic skin (Church and Levi-Schaffer 1997; Sherman and others 1999). Human and rodent mast cells constitutively store IL-4 (Brody and Metcalfe 1998; Sherman and others 1999). Furthermore, the majority of IL-4^+ cells in human (Kobayashi and others 2000) and canine (Sinke and others 1998) atopic skin appear to be mast cells.

1.9.6 The role of SCF and *c-Kit* in atopic dermatitis

SCF/*c-Kit* interactions enhance *in vitro* survival, proliferation, granularity and cytokine expression of canine (Brazís and others 2000) and human (Welle 1997) mast cells. In human atopic dermatitis there is a correlation between SCF expression, mast cell numbers and dermal histamine content (Rossi and others 1998; Galli and others 1999). A recent study also found higher levels of SCF in atopic compared to healthy canine skin (Hammerberg and others 2001).

SCF can potentiate IgE dependent degranulation in rat mast cells (Hill and others 1996) and both IgE dependent and independent mast cell activation in atopic humans (Brody and Metcalfe 1998; Galli and others 1999; Kanbe and others 1999). SCF enhanced FcεRI mediated degranulation and cytokine synthesis in canine mast cells, but was not capable of triggering mast cell activation by itself (Brazís and others 2000). SCF/*c-Kit* interaction also up regulates integrin receptor expression in human mast cells. This increases mast cell adherence to the dermal matrix (Brody and Metcalfe 1998), which is important in mast cell recruitment and activation during inflammation (Williams and Galli 2000).

1.10 Cutaneous micro-organisms and atopic dermatitis

1.10.1 Staphylococci and superantigens

Microbial colonisation and infection play a significant role in atopic dermatitis. Up to 80% of human cases are colonised with *Staphylococcus aureus* (Bunikowski and others 1999). Colonisation of lesional skin can reach 90%, but numbers of staphylococci are also elevated on non-lesional skin (Skov and Baadsgaard 2000). Furthermore, colonisation correlates with the severity of clinical signs in human atopic dermatitis (Herz and others 1998). *S. intermedius* can be recovered from mucocutaneous sites in many healthy dogs, but atopic dogs exhibit both higher counts and greater adherence of staphylococci to keratinocytes (DeBoer and Marsella 2001).

Severe human atopic dermatitis is associated with superantigen producing strains (Zollner and others 2000). 55-65% of *S. aureus* strains produce superantigens including staphylococcal enterotoxin (SE)A, SEB, SEC and toxic shock syndrome toxin (TSST)-1 (Bunikowski and others 1999; Zollner and others 2000). Toxic shock syndrome survivors are predisposed to atopic dermatitis (Michie and Davis 1996; Torres and others 1998) and SEB induces and exacerbates lesions (Skov and others 2000).

Superantigens activate T-cells bearing the appropriate Vβ TCR chain by directly linking MHCII and TCR molecules in a non-antigen specific manner (Huston 1997). This activates a polyclonal pool of $CD4^+$ and $CD8^+$ cells 10-100 times larger than that activated by specific allergens (Bunikowski and others 1999; Skov and Baadsgaard 2000). In humans there is an association between these Vβ elements and house dust mite specific TCRs (Herz and others 1998).

Staphylococcal superantigens are strongly pro-inflammatory. They activate mast cells and basophils *in vitro* (Bunikowski and others 1999), up regulate expression of IL-1,

GM-CSF, IL-12 and TNFα from mononuclear cells and T-cells (Strickland and others 1999; Leung 2000). They also induce CD23 and MHCII expression on keratinocytes, monocytes, dendritic cells and T-cells (Herz and others 1998; Skov and others 2000). In humans and mice, staphylococcal superantigens induce CLA expression on T-cells (Abbas and others 1996; Strickland and others 1999) and up regulate endothelial intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, enhancing leucocyte adhesion and migration (Herz and others 1998). TSST-1 induces MHCII and co-stimulatory molecules on B-cells, augmenting IgE production (Hofer and others 1999).

SEA, B, C, D, E and TSST-1 have been detected in up to 40% of *S. intermedius* strains isolated from dogs (Adesiyun and Usman 1983; Hirooka and others 1988), but a role in canine atopic dermatitis remains to be established.

Superantigens may also act as conventional allergens. Serum superantigen-specific IgE is found in 50-80% of human atopic patients and has been linked to clinical severity in some studies (Herz and others 1998; Bunikowski and others 1999; Leung 2000; Breuer and others 2000), but not others (Zollner and others 2000). Anti-staphylococcal IgE has been identified in canine atopic dermatitis complicated by recurrent staphylococcal pyoderma (Morales and others 1994), but its significance is unclear.

1.10.2 Malassezia and atopic dermatitis

A proportion of human patients with atopic dermatitis develop a hypersensitivity response to cutaneous *Malassezia* (Nordvall and Johansson 1990; Broberg and others 1992; Jensen-Jarolim and others 1992). High serum levels of *Malassezia* specific IgE

found in patients with atopic dermatitis were not found in sera from either healthy patients or non-atopic patients with *Malassezia* dermatoses (Wessels and others 1991). Immediate and late phase skin reactivity, and lymphocyte proliferation in response to *Malassezia* extracts were also demonstrated in atopic but not non-atopic patients (Kieffer and others 1990; Nordvall and Johansson 1990; Rokugo and others 1990; Wessels and others 1991; Tengvall Linder and others 1998). However, no evidence for superantigen activity or polyclonal activation of T-cells has been found (Johansson and others 1999).

Malassezia dermatitis and otitis are well recognised in canine dermatology and frequently complicate atopic dermatitis (Mason and Evans 1991; Plant and others 1992; Bond and others 1996). Intradermal test reactivity to a crude *Malassezia* extract has been observed in atopic, but not healthy, dogs (Morris and others 1998). Atopic dogs with *Malassezia* dermatitis also developed larger wheals than those without. Recent findings have also shown that atopic dogs have significantly higher serum levels of *Malassezia* specific IgE than healthy dogs, which are not dependent on the presence of elevated cutaneous numbers of *Malassezia* (Nuttall and Halliwell 2001; Chen and others 2002). Together, these data suggest that a proportion of atopic dogs are sensitised to *Malassezia* antigens. Despite this, recent findings suggest that PBMCs isolated from atopic and healthy dogs have similar responses to *Malassezia* extracts *in vitro* (Morris and others 2002).

1.11 Skin homing in atopic dermatitis

The route of allergen exposure may determine the clinical spectrum of atopic reactions - inhaled allergens trigger rhinitis and asthma, whilst percutaneous exposure

triggers dermatitis (Friedmann 1999; Wistokat-Wulfing and others 1999). Human patients often have respiratory symptoms as well as dermatitis, but atopy patch tests are rarely positive in patients without dermatitis (Wistokat-Wulfing and others 1999).

In dogs, clustering of IgE^+ Langerhans' cells, T-cells and eosinophils within the epidermis is consistent with epidermal antigen capture (Olivry and others 1997). Affected skin is predominantly ventral and sparsely haired, which could also facilitate allergen uptake. Furthermore, atopy patch tests closely resemble late phase reactions (Rutten and others 2001) and genetically predisposed dogs develop both specific IgE and skin lesions following cutaneous application of allergens (McCall and others 2002). In contrast, nasal allergen exposure generally fails to exacerbate the disease (Olivry and Hill 2001).

Why respiratory disease is uncommon in dogs is unclear. Their complex nasal turbinates may prevent allergen penetration to the lower airways or there may be differential expression of adhesion molecules or inflammatory mediators in skin and mucosal sites. The propensity to develop dermatitis may also depend on the level of CLA expression and relative proportions of skin and respiratory mucosa homing T-cells. In human atopic dermatitis allergen specific cells are CLA^+, whereas they are predominantly CLA^- in atopics without dermatitis (Leung 2000). Cutaneous T-cell attracting chemokine (CTACK or CCL27) and IL-16, both important in CLA^+ T-cell recruitment, are up regulated in human atopic skin (Leung 2000).

IL-1, IL-4 and TNFα increase endothelial expression of E-selectin (the CLA ligand), ICAM-1 and VCAM-1 in humans (Leung 1999). ICAM-1 is also expressed on keratinocytes in human (Leung 2000) and canine (Olivry and others 1999a) atopic dermatitis. P-selectin, another endothelial ligand for leucocyte adhesion molecules, is up

regulated in canine inflammatory skin diseases, including atopic dermatitis (Chénier and Doré 1998).

Chemokines such as RANTES (regulated on activation normal T-cell expressed and secreted), MCP-4 (macrophage chemotactic protein 4) and eotaxin are also up regulated in atopic skin. Furthermore, their receptors, including CCR3, are preferentially expressed on T_{H2} cells and eosinophils (Leung 2000).

1.12 Tolerance in healthy individuals

It is unlikely that T_{H1} differentiation inhibits T_{H2} responses in healthy individuals, as allergen induced cell-mediated inflammation is absent and T_{H1} cytokines participate in chronic atopic dermatitis. Healthy individuals must, therefore, develop cutaneous tolerance (Borish and Rosenwasser 1997). Experimental models of autoimmune and allergic diseases suggest that the immunosuppressive cytokines IL-10 and TGFβ1 are important in tolerance (Yeo and Lamb 1995; Muraille and Leo 1998; Massey and others 2001).

1.12.1 T-regulatory cells

IL-10 and $TGF\beta_1$ induce differentiation of allergen specific T-regulatory cells (T_R, T_{reg}, T_{H3} or T-suppressor cells) that down regulate inflammatory responses (Yeo and Lamb 1995; Kapsenberg and others 1998; Mason and Powrie 1998; Schmidt-Weber and others 1999; Hoyne and others 2000; Koulis and Robinson 2000). T-regulatory cells could inhibit T_{H1} and T_{H2} activation by direct interaction of the notch-delta signalling pathway (Hoyne and others 2000), which may be a mechanism for acquiring tolerance to different allergens presented by the same APC ('linked suppression').

1.12.2 The role of TGFβ

The TGFβ family of cytokines are involved in cell growth and development, regulating inflammation and tissue remodelling, and initiating early immune responses (Ling and Robinson 2002). They are produced by a wide range of cells and have a variety of stimulatory and inhibitory effects on T-cell differentiation, activation, proliferation, apoptosis and APC activity depending on the cell types and their state of differentiation and activation (Cottrez and Groux 2001). TGFβ1 is particularly anti-inflammatory - knockout mice exhibit spontaneous and widespread inflammation that is rapidly fatal (Sumiyoshi and others 2002). It is thought to suppress a variety of inflammatory disorders and appears to be important in the regulation and resolution of inflammatory reactions (Ling and Robinson 2002). TGFβ1 inhibits B and T-cell activation and inflammatory cytokine production (Lingnau and others 1998; Skok and others 1999), mast cell development and degranulation (Mekori and Metcalfe 1999), as well as eosinophil survival, expression of adhesion molecules and IgE production (Sumiyoshi and others 2002). TGFβ1 activity is dependent on TGFβ receptor II expression, which is induced by IL-10 (Cottrez and Groux 2001).

1.12.3 The role of IL-10

IL-10 is produced by a wide variety of cells including keratinocytes, T_H cells, B-cells, mast cells, eosinophils, monocytes and APCs in response to IL-2, IL-4, IL-6 and IL-12 (Borish 1998; Muraille and Leo 1998; Sato and others 1998; Koulis and Robinson 2000). IL-10 inhibits production of pro-inflammatory cytokines and chemokines by macrophages, neutrophils, eosinophils, mast cells, lymphocytes, keratinocytes and endothelial cells, as well as immunoglobulin synthesis, MHCII and co-stimulatory

molecule expression, antigen presentation, and chemotaxis (Borish 1998; Muraille and Leo 1998; Asadullah and others 1999; Schmidt-Weber and others 1999; Skok and others 1999; Koulis and Robinson 2000).

Decreased IL-10 expression is associated with inflammatory diseases. IL-10 deficient mice develop severe skin, gut and airway inflammation (Borish 1998; Tournoy and others 2000). There is also decreased IL-10 in skin and PBMCs from atopic compared to healthy children (Koning and others 1997b). Furthermore, it has been suggested that successful immunotherapy is associated with up regulation of IL-10 expression (Smith and Sly 1998; Akdis and Blaser 1999).

IL-10 is a potent inhibitor of IL-12 (Muraille and Leo 1998; Asadullah and others 1999; Koulis and Robinson 2000) and could also potentiate T_{H2} responses. Increased levels of IL-10 have been found in human atopic skin, airways and PBMCs after allergen challenge (Laberge and others 1998; Sato and others 1998; Hanifin and Chan 1999; Koulis and Robinson 2000) and in a mouse model of atopic dermatitis (Vestergaard and others 1999).

The kinetics of IL-10 expression may govern its balance of biological activities. In atopic humans and a mouse model, IL-10 is constitutively expressed in healthy skin, but only during the late phase response in atopic skin (Muraille and Leo 1998; Wang and others 1999), probably in response to IL-12 and IFNγ production. Late expression of IL-10 in mice may contribute to atopic inflammation by supporting IgE and IgG production by IL-4 stimulated B-cells, and mast cell differentiation and activity (Sato and others 1998; Asadullah and others 1999; Tournoy and others 2000). In parasitised humans, IL-10 is thought to both support parasite-directed T_{H2} responses and suppress systemic allergic responses (van den Biggelaar and others 2001).

1.13 The development of sensitisation *versus* tolerance

Sensitisation *versus* tolerance is probably determined by allergen exposure at critical time points (Holt and others 1998). For instance, in high IgE responder beagles, sensitisation by seven days of age generates a persistent high IgE response, whereas there is only a transient peak after sensitisation at 3-4 months (de Weck and others 1997). Recent interest has focused on why allergen exposure leads to sensitisation in atopic individuals and tolerance in healthy individuals.

1.13.1 T_{H2} polarisation in human infants

Virtually all human infants develop T_{H2} polarised responses to environmental allergens *in utero* (Holt 1995; Prescott and others 1998). Cord blood mononuclear cells typically express high levels of IL-4, IL-5 and IL-10, and low levels of IFNγ (Prescott and others 1997), although T_{H2} differentiation is particularly marked in children with atopic parents (Piccinni and others 1996; Prescott and others 1997). Inhibition of T_{H1} differentiation by IL-4, IL-10, PGE_2 and progesterone might protect the foeto-placental unit from cell mediated immune responses (Holt and Macaubas 1997; Prescott and others 1997). Tolerance is associated with post-natal maturation of T_{H1}-type immunity (Prescott and others 1999). In contrast, T_{H2} responses persist in atopic children (Holt and others 1998; Prescott and others 1999), although only a proportion develop clinical signs (Holt 1995).

1.13.2 The hygiene hypothesis in T_{H1} development

Lack of exposure to micro-organisms and their products (e.g. fungi, gut flora mycobacteria, lipopolysaccharides, endotoxins, etc.) could delay post-natal development of

T_{H1} responses and APC maturation (Holt 1995; Holt and Macaubas 1997; Hopkin 1997; Romagnani and others 1997; Holt 1998; Lewis 2000). Bacterial CpG DNA motifs are particularly potent inducers of T_{H1} responses (Parronchi and others 2000). Decreased exposure to gram-negative endotoxin in house dust was also associated with positive skin tests to environmental allergens and reduced numbers of $CD4^+$ $IFN\gamma^+$ cells in human infants (Gereda and others 2000). Furthermore, exposure to farm animals and consumption of more 'live' or 'natural' compared to processed foods confers protection from atopy, although the effect was most marked for out-door allergens (Kilpelainen and others 2000; Riedler and others 2000). An inverse correlation of atopic diseases and exposure to hepatitis A, *Helicobacter pylori* and *Toxoplasma gondii*, but not airborne viruses, suggests that gut flora or gut-borne infections are particularly important in T_{H1} development and tolerance (Matricardi and others 2000). Similar studies have not been performed in dogs, although viral vaccines were shown to enhance IgE production (Frick and Brooks 1983). Interestingly though, many dogs in Western societies live in relatively sterile indoor environments, are fed processed food and receive multiple vaccinations and antibiotic courses.

1.13.3 Pollution and T_{H2} polarisation

Pollution has also been associated with T_{H2} responses (Nel and others 1998). However, atopic sensitisation is less common in the more polluted, but less affluent Baltic states compared to Scandinavia (Erb 1999). Furthermore, atopic diseases in East Germany increased after reunification, despite decreased pollution (Erb 1999; Von Ehrenstein and others 2000). Vehicle, particularly diesel, emissions, however, are

greater in affluent societies. These have been associated with T_{H2} polarisation and may be more important than overall pollution (Polosa 1999; Diaz-Sanchez 2000).

1.14 Aims and objectives of this project

1.14.1 Introduction

Compared to humans and mouse models, our understanding of canine atopic dermatitis is relatively poor. Until recently, most publications have concentrated on the clinical signs, skin testing, serology and treatment options. The aim of these studies, therefore, is to further characterise the immunological mechanisms that underlie canine atopic dermatitis. Individual studies will look in detail at the clinical features of atopic dermatitis, allergen specific IgE responses, major and minor *Dermatophagoides* allergens, PBMC responses and the expression of T_{H1}, T_{H2} and immunosuppressive cytokines in skin and PBMCs.

1.14.2 Clinical features of skin test positive and skin test negative canine atopic dermatitis

Distinct allergic and non-allergic forms of human atopic dermatitis appear to exist (Akdis and others 1999a). It is, however, unclear if there are two forms of canine atopic dermatitis, as the clinical and histopathological features of allergen reactive and non-reactive dogs have not been studied in detail. Clinical and histopathological data from skin test positive and negative dogs with a clinical diagnosis of atopic dermatitis were therefore compared.

1.14.3 Major and minor *Dermatophagoides* allergens in canine atopic dermatitis

It is clear that *Dermatophagoides* species house dust mites are the most important allergens in both human and canine atopic dermatitis (Cooper 1994; Sture and others 1995). Previous studies suggest that different *Dermatophagoides* allergens are recognised by dogs and humans (Noli and others 1996; McCall and others 2000), although other findings refute this (Masuda and others 1999).

These studies all used different antisera, allergen extracts and protocols. The allergen content of extracts varies depending on the source, mite life cycle and proportion of mites to media (Le Mao and others 1998). Different antisera can vary in specificity and sensitivity (Steward and Male 1998). Some protocols, furthermore, involve reducing agents that can denature IgE epitopes (Lombardero and others 1990).

Crude and purified *Dermatophagoides* extracts obtained from a number of sources were, therefore, compared using reducing and non-reducing sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) gels. Major and minor allergens were identified and compared by probing reduced and non-reduced western blots of each extract with monoclonal and polyclonal anti-canine IgE antibodies.

1.14.4 Isolation and culture of canine PBMCs

PBMC cultures are routinely used to investigate lymphocyte responses to specific allergens (Steward and Male 1998). A key aim of this study is to investigate PBMC responses to *Dermatophagoides* allergens in atopic and healthy dogs. However, to do so requires a robust technique for the isolation and culture of canine PBMCs. Density centrifugation over 1.077 g/L ficoll has been used to isolate canine PBMCs in a

number of studies (Kristensen and others 1982a; Martínez-Moreno and others 1995; Corato and others 1997; Poitout and others 1997), but other papers report that this results in granulocyte contamination (Letwin and Quimby 1987; Rivas and others 1995). These authors used carbonyl iron to eliminate the neutrophils, although this could also deplete the mononuclear phagocytic cells necessary for antigen presentation in PBMC cultures (Krakowka and Ringler 1985).

A preliminary study was therefore undertaken to compare PBMC yield and culture after density centrifugation with and without incubation with carbonyl iron.

1.14.5 PBMC responses to *Dermatophagoides* allergens

Canine atopic dermatitis has traditionally been viewed as an IgE/mast cell mediated disease. IgE production, however, is dependent on B-cell interaction with allergen specific $CD4^+$ T_H cells (Lydyard and Grossi 1998). Infiltration of $CD4^+$ and $CD8^+$ lymphocytes into lesional skin, furthermore, is observed in both atopic dogs (Olivry and others 1997) and humans (Leung 2000). Several studies in humans have demonstrated PBMC proliferation to crude *Dermatophagoides* extracts and purified allergens (Leung 2000). Despite this, investigation of PBMC responses in atopic dogs has been limited. One unpublished report suggested that PBMCs from atopic dogs proliferated in response to stimulations with crude *D. pteronyssinus* extract and major allergens identified on western blots, but not to Der p 1 or Der p 2 (Shaw 2000). Responses to *D. farinae*, however, were not investigated.

The aim of this study was to determine if allergen specific T-cells are 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 and skin test negative

to *D. farinae*, and healthy dogs. The responses of PBMCs isolated from skin test positive dogs to major and minor allergens purified from *D. farinae,* and crude extracts of *D. farinae*, *D. pteronyssinus* and *D. microceras* were also compared.

1.14.6 Characterisation of cytokine expression in canine atopic dermatitis

Several studies have observed T_{H2} polarisation in the skin and PBMCs of atopic humans (van der Heijden and others 1991; van Reijsen and others 1992; Neumann and others 1996; Koning and others 1997a; Koning and others 1997b) and mouse models of atopic dermatitis (Spergel and others 1999; Vestergaard and others 1999). Despite this, T_{H1}-type cytokines have been detected in chronic lesions (Werfel and others 1996) and dominate atopy patch test sites after 48 hours (Thepen and others 1996). In contrast, tolerance in healthy individuals may be mediated by the immunosuppressive cytokines IL-10 and TGFβ1 (Muraille and Leo 1998; Hoyne and others 2000; Koulis and Robinson 2000). Studies of cytokine expression in canine atopic dermatitis are limited. IL-4 has been detected in lesional atopic skin by immunohistochemistry and Il-4 mRNA by RT-PCR (Sinke and others 1998). Another study using non-quantitative RT-PCR demonstrated a T_{H2}-type cytokine pattern in 25% of atopic and a T_{H1}-type pattern in 25% of healthy skin samples (Olivry and others 1999b).

Several authors suggest that up regulation of the SCF/*c-Kit* pathway enhances mast cell development and cytokine expression in both human (Brody and Metcalfe 1998; Galli and others 1999) and canine (Brazís and others 2000) atopic dermatitis. In human atopic dermatitis there is a correlation between SCF expression, mast cell numbers and dermal histamine content (Rossi and others 1998; Galli and others 1999).

We, therefore aimed to use semi-quantitative RT-PCR to compare the expression of mRNA encoding the T_{H1}-type cytokines IFNγ, TNFα, IL-2 and IL-12, the T_{H2}-type cytokines IL-4 and IL-6, the immunosuppressive cytokines IL-10 and TGFβ1, as well as SCF and *c-Kit* in atopic and healthy canine skin, as well as resting, mitogen and specific allergen stimulated PBMCs isolated from atopic and healthy dogs.

1.15 Conclusions

The significance of these studies is threefold. Firstly, they should advance our understanding of canine atopic dermatitis, a very common and distressing condition. Secondly, canine atopic dermatitis could be a valuable, spontaneous model for studying the human disease. Finally, establishing the immunological mechanisms underlying tolerance will allow us to further develop canine atopic dermatitis as a powerful model to explore new approaches to therapy in dogs and humans. Identifying the key mediators will also allow us to utilise molecular techniques to investigate genetic polymorphisms in between atopic and healthy dogs.

2. Materials and methods

2.1 Atopic and healthy dogs – inclusion criteria and clinical findings

2.1.1 Source of dogs

Dogs with atopic dermatitis (n=45) were recruited from the dermatology clinic at the University of Edinburgh Hospital for Small Animals, Easter Bush Veterinary Centre, Easter Bush, Midlothian, EH25 9RG, UK. Control samples were taken from healthy dogs, either belonging to staff and students (n=10), or presented for euthanasia at the University of Edinburgh Hospital for Small Animals (n=16). Euthanasia was performed with intravenous sodium pentobarbitone (Euthetal®; Mérial, Harlow, UK.).

All procedures were performed with Home Office approval (project licence no. 60:2336) and were subject to the Animals (Scientific Procedures) Act 1986. Informed consent from the owners of all animals, except the euthanasia cases, was obtained prior to entry into the study (Figure 2.1 a and b). All animals, with the exception of the euthanasia cases, were returned to the owners on completion of the procedure (Figure 2.2). Permission to take samples from the euthanasia cases was obtained from each owner. All procedures on these animals were carried after effective euthanasia, as assessed by the lack of cardiac activity, respiration and central reflexes.

2.1.2 Inclusion criteria for the healthy control dogs

None of the healthy dogs had a history of pruritic skin disease or systemic conditions likely to affect immune function. All the dogs were clinically ascertained to be free of lesions at the time of sampling. No topical or systemic anti-inflammatory medications were given for at least three weeks (eight weeks for injectable depot preparations) prior to examination.

Figure 2.1a - Informed consent form for dogs with atopic dermatitis

SURNAME OF APPLICANT:

>> <<

OFFICE USE ONLY

PPL NUMBER	DATE
60 2336	19 SEP 1998

DATE OF ISSUE OF LICENCE:

HOME OFFICE RECEIVED

Informed consent 1 - Atopic Dogs

Canine Allergy Study

1. I understand that in order to advance our understanding of allergic skin diseases in dogs, and to develop new treatments, there is a need to determine how dogs respond to allergens (substances such as house dust mites that lead to allergies).

2. This can be investigated by injecting allergens into the skin (an intradermal allergy test); by investigating how cells in the blood respond to allergens (blood test); and by determining how cells in the skin respond to allergies (skin biopsy).

3. I understand that the protocol involved in this study involves three tests: an intradermal allergy test, a blood test and skin biopsies.

4. I understand that the intradermal allergy test involves mild sedation with xylazine which is a widely used drug in veterinary practice. A low dose will be used and, apart from the possibility of vomiting, the incidence of adverse reactions is exceedingly rare. A patch of hair (approximately 15cm x 25 cm) will be shaved off, and 60-70 small intradermal injections given. I understand that no adverse reactions, apart from possibly mild irritation for 1-2 days at one or more of the sites, are to be anticipated

5. I understand that a blood sample will be obtained, but no adverse effects are to be anticipated.

6. I understand that a small volume of local anaesthetic will be injected under the skin at a number of sites (up to 5). Small skin biopsies will be obtained with a punch and a single suture will be placed in the skin afterwards. I understand that skin biopsies do not appear to cause adverse effects in dogs. Some mild discomfort may be felt at the injection site of the local anaesthetic but this is very transient. The biopsy procedure itself causes no discomfort. I understand that there are virtually no clinical problems associated with taking skin biopsies in dogs. I also understand that if my dog removes its own sutures, the wounds will normally heal without any problems and are not likely to require further treatment. However, if any problems occur, I will contact the clinic at the Royal (Dick) School of Veterinary Studies for advice.

7. I understand that the results of the intradermal allergy test and some of the biopsies will be used to make a diagnosis of my dog's skin disease. I also understand that the blood sample and some of the biopsies will be used for research purposes to further investigate allergic skin diseases in dogs. In view of the importance of the study in helping dogs with skin disease, I consent to the above procedures.

8. I understand that my own dog may not benefit immediately from this research, but the results of the study may benefit mine, or other dogs, in the future.

9. I understand that the protocol for this study has been approved by the Home Office.

Signed _____ Date _____

Figure 2.1b - Informed consent form for healthy dogs

SURNAME OF APPLICANT:

>> <<

OFFICE USE ONLY

PPL NUMBER	DATE
60 2335	18 SEP 1998

DATE OF ISSUE OF LICENCE:

HOME OFFICE RECEIVED

Informed consent 2 - Normal dogs

Canine Allergy Study

1. I understand that in order to advance our understanding of allergic skin diseases in dogs, and to develop new treatments, there is a need to determine how normal dogs respond to allergens (substances such as house dust mites that lead to allergies).

2. As far as I am aware, my pet does not, and has not suffered from allergic skin disease.

3. I understand that the study protocol involves mild sedation with xylazine which is a widely used drug in veterinary practice. A low dose will be used and, apart from the possibility of vomiting, the incidence of adverse reactions is exceedingly rare.

4. I understand that a small area (approximately 4cm x 25 cm) of hair will be shaved off, and 12-15 small intradermal injections given.

5. I understand that no adverse reactions, apart from possibly mild irritation for 1-2 days at one or more of the sites, are to be anticipated.

6. I understand that a blood sample will be obtained, but no adverse effects are to be anticipated.

7. In view of the importance of the study in helping dogs with skin disease, I consent to the above procedures.

8. I understand that as my own dog is healthy, it will not benefit from the study unless it should later develop allergies.

9. I understand that the protocol for this study has been approved by the Home Office.

Signed _____ Date _____

Figure 2.2 - Experimental animal release form

DEPARTMENT of VETERINARY CLINICAL STUDIES
Royal (Dick) School of Veterinary Studies

The University of Edinburgh
Hospital for Small Animals
Easter Bush Veterinary Centre
Roslin Midlothian
EH25 9RG

Fax 0131 650 7652
Telephone 0131 650 7650
or direct dial 0131 650

Project Licence no. - 60/2336

Date - .....
Owner's name - .....
Animal's name - .....
Clinic no. - .....
Project case no. - .....

The afore-mentioned animal has been certified as fit for release to the owner.

Signed

Emma Keeble BVSc CertZooMed MRCVS
Named Veterinary Surgeon

Gidona Goodman BSc MSc(Vetmed) MSc MRCVS
Named Veterinary Surgeon

2.1.3 Inclusion criteria for the dogs with atopic dermatitis

A clinical diagnosis of atopic dermatitis was based on a history of chronic, perennial pruritus that was responsive to glucocorticoids, but non or only partially responsive to antimicrobial and antiparasitic treatment, and exclusion of other causes of pruritus (Willemse 1986; Nuttall and others 1998; DeBoer and Hillier 2001).

Multiple coat brushings and skin scrapings were used to eliminate the possibility of an ectoparasite infestation. All dogs were placed on a rigorous flea control programme for a minimum of six weeks using topical fipronil (Frontline®; Mérial) or selamectin (Stronghold®; Pfizer Animal Health, Sandwich, UK). Other trial antiparasitic therapy with an appropriate product was employed where there was a high index of suspicion, despite a lack of diagnostic clinical findings. Dogs that experienced complete resolution of clinical signs after treatment were excluded.

All dogs underwent a six-week, home cooked or commercial diet trial to eliminate food intolerance. Novel single carbohydrate and protein sources were selected on the basis of previous dietary history. Provocative exposure to the normal diet was used to confirm food intolerance in dogs that clinically improved on the trial diet. Dogs that completely responded to the diet trial were excluded.

Staphylococcal pyoderma was managed with appropriate systemic antibiotics and topical treatment with either 2.5% benzoyl peroxide (Paxcutol®; Virbac, Bury St.Edmonds, UK) or 10% ethyl lactate (Etiderm®; Virbac). *Malassezia* dermatitis was treated with a 2% miconazole/2% chlorhexidine shampoo (Malaseb®; Leo Animal Health, Princes Risborough, UK). Otitis externa was treated with topical polyvalent and/or systemic anti-microbial products according to the requirements of each

case. Dogs that experienced a complete resolution of pruritus after antimicrobial treatment were excluded.

No topical or systemic anti-inflammatory or antimicobial medication was given for at least three weeks (eight weeks for injectable depot preparations) before examination.

2.1.4 Scoring of clinical lesions

Clinical signs of erythema, papules, scaling, seborrhoea, hyperpigmentation and lichenification at multiple body sites were subjectively assessed by a single investigator (T.J. Nuttall) and scored from 0-5, with 0 defined as lesion free (Figure 2.3). Erythema was defined as reddening of the skin that could be blanched by digital pressure. Papules were defined as small, focal and erythematous raised lesions without gross evidence of crusting or pustule formation. The degree of scaling was based on the amount of cornified debris accumulated on the hairs and skin surface. Seborrhoea in this study was used to denote excessive greasiness of the skin and coat. Lichenification was defined as the degree of skin thickening and fissuring compared to unaffected skin, ignoring pressure point calluses. Hyperpigmentation referred to darkening of the skin compared to the colour of unaffected skin in each individual.

The presence or absence of pruritus at any body site was determined from the clinical history taken from the owner by the clinician in charge of each case (P.B. Hill, A.W. Carter, P. Forsythe, S. Colombo, R.E.W. Halliwell or K.L. Thoday; Dermatology Service, University of Edinburgh Hospital for Small Animals). Because of the subjective nature of these data no attempt to assess the degree of pruritus was made.

The clinician in charge of each case also determined the presence or absence of staphylococcal pyoderma and *Malassezia* dermatitis at each body site. The presence

of pustules, crusted papules, epidermal collarettes and, where performed, typical staphylococcal organisms on Diff-Quik® (Dade AG; Düdingen, Switzerland) stained cytological preparations were used to diagnose staphylococcal pyoderma. The presence of erythema, seborrhoea, lichenification and hyperpigmentation together with microscopic observation of greater than five organisms per x400 field on Diff-Quik® stained tape-strips or smears was used to diagnose clinically significant *Malassezia* dermatitis and otitis. As multiple investigators assessed these data, the severity or degree of infection was not recorded.

Figure 2.3 - Clinical record sheet

Investigation into Canine Atopy

Case no. - .....
Date - .....

Clinic no. - .....
Name - .....
Breed - .....
Age - .....
Sex - .....
Clinician - .....

Samples

- Group - .....
- Sedation - .....
- Blood - .....
- Biopsies - *Sites/Lesions* - .....
  .....
  .....
- Intradermal Skin Test - .....

Sites	Pruritus	Erythema	Papules	Scaling	Seborrhoea	Pigmentation	Lichenification	Malassezia	Pyodermia
Muzzle									
Head									
Left ear									
Right ear									
Dorsal neck									
Ventral neck									
Dorsal body									
Left axilla									
Right axilla									
Left flank									
Right flank									
Ventral body									
Left groin									
Right groin									
Perineum									
Tailhead									
Left fore:									
Upper limb									
Distal limb									
Metacarpals									
Dorsal foot									
Ventral foot									
Right Fore:									
Upper limb									
Distal limb									
Metacarpals									
Dorsal foot									
Ventral foot									
Left hind:									
Upper limb									
Distal limb									
Metatarsals									
Dorsal foot									
Ventral foot									
Right hind:									
Upper limb									
Distal limb									
Metatarsals									
Dorsal foot									
Ventral foot									

2.1.5 Intradermal skin tests

All dogs with a clinical diagnosis of atopic dermatitis were intradermally tested with 56 allergens (Table 2.1) as follows. Dogs were sedated with 0.15mg/kg xylazine (Virbaxyl®; Virbac) injected intramuscularly. Hair was clipped from the lateral flank and 0.05ml of each allergen extract, 0.1mg/ml histamine (positive control) and saline with phenol diluent (negative control) were injected intradermally *via* a 25-gauge needle. The degree of swelling, erythema and induration at each injection site were subjectively assessed after 20 minutes. The saline and histamine wheals were arbitrarily assigned scores of 0 and +4 respectively. The degree of erythema and swelling at each test site was subjectively scored 0, +1, +2, +3 or +4 in comparison to the negative and positive control sites. Reactions greater than or equal to +2 were considered positive (Nuttall and others 1998).

The intradermal skin tests were performed and assessed according to these standard criteria by the clinician in charge of each case (P.B. Hill, A.W. Carter, P. Forsythe, S. Colombo, R.E.W. Halliwell or K.L. Thoday; Dermatology Service, University of Edinburgh Hospital for Small Animals). These criteria provide a robust differentiation between positive and negative sites (T.J. Nuttall and K.L. Thoday; unpublished data) but the use of multiple clinicians precluded any statistical analysis of the test sites. Serum from dogs with negative skin tests was submitted to a commercial laboratory for measurement of allergen specific IgE using recombinant canine FcεRIα (Heska Allercept® provided by Axiom Laboratories, Teignmouth, UK). Dogs with a negative skin test but positive serology were excluded from the study.

Table 2.1 – Intradermal test allergens

Greer – Greer laboratories, Lenoir, NC, USA; ARTU – Artuvetrin, Leyden, The Netherlands; PNU – protein nitrogen units; NE – nitrogen equivalents.

Allergen extract	**Source**	**Concentration**
Dust and dust mites		
Dermatophagoides farinae	Greer	100μg/ml
Dermatophagoides pteronyssinus	Greer	100μg/ml
Tyrophagus putresceantiae	ARTU	100 NE/ml
Acarus siro	ARTU	100 NE/ml
Euroglyphus maynei	ARTU	100 NE/ml
House dust	Greer	10μg/ml
Epithelia		
Cat epithelia	Greer	500PNU/ml
Human epithelia	ARTU	500PNU/ml
Mixed feathers	Greer	500PNU/ml
Cotton linters	Greer	250PNU/ml
Horse epithelia	Greer	500PNU/ml
Sheep epithelia	Greer	500PNU/ml
Insects		
Blackfly	Greer	500PNU/ml
Flea	Greer	500PNU/ml
Mixed moths	Greer	500PNU/ml
Mosquito	Greer	500PNU/ml
American cockroach	Greer	500PNU/ml
German cockroach	Greer	500PNU/ml
Culicoides midge	Greer	500PNU/ml
Tree pollens		
Elder	ARTU	1000 NE/ml
Box elder	Greer	1000PNU/ml
Elm	ARTU	1000 NE/ml
American elm	Greer	1000PNU/ml
Alder	ARTU	1000 NE/ml
Tag alder	Greer	1000PNU/ml
Ash	ARTU	1000 NE/ml
White ash	Greer	1000PNU/ml
Beech	ARTU	1000 NE/ml
American beech	Greer	1000PNU/ml
Birch	ARTU	1000 NE/ml
White pine	Greer	1000PNU/ml
White oak	Greer	1000PNU/ml
Eastern sycamore	Greer	1000PNU/ml

Allergen extract	Source	Concentration
Weed pollens		
Red clover	Greer	1000PNU/ml
Lamb's quarter	Greer	1000PNU/ml
Chrysanthemum spp.	Greer	1000PNU/ml
Yellow dock	Greer	1000PNU/ml
Jerusalem oak	Greer	1000PNU/ml
Common mugwort	Greer	1000PNU/ml
Brassica spp.	Greer	1000PNU/ml
English plantain	Greer	1000PNU/ml
Grass pollens		
Couch	Greer	1000PNU/ml
Red top	Greer	1000PNU/ml
Kentucky blue	Greer	1000PNU/ml
Meadow fescue	Greer	1000PNU/ml
Perennial rye	Greer	1000PNU/ml
Orchard	Greer	1000PNU/ml
Sweet vernal	Greer	1000PNU/ml
Timothy	Greer	1000PNU/ml
Yorkshire fog	Greer	1000PNU/ml
Moulds		
Aspergillus	Greer	1000PNU/ml
Botrytis	Greer	1000PNU/ml
Altenaria	Greer	1000PNU/ml
Penicillium	Greer	1000PNU/ml
Oak smut	Greer	1000PNU/ml
Barley smut	Greer	1000PNU/ml

2.2 Sample collection

2.2.1 Blood samples for serum collection

10-20 ml blood samples for serum were collected into plain glass tubes by jugular venepuncture in live dogs or cardiac puncture immediately after euthanasia. The tubes were kept at room temperature for 1-2 hours to allow clot retraction. The serum was then separated by centrifugation at 2200g for five minutes and stored at -20°C.

2.2.2 Blood samples for PBMC isolation

20-40 ml blood samples for PBMC isolation were collected into 1.2% tri-sodium citrate in sterile 50ml polypropylene centrifuge tubes (Fisher Scientific, Loughborough, UK) by jugular venepuncture in live animals or cardiac puncture immediately after euthanasia. Samples were kept on ice and processed (Chapter 2.6) within two hours.

2.2.3 Tissue samples from dogs with atopic dermatitis

Skin samples were taken whilst the dogs were sedated for intradermal testing (Chapter 2.1.5) as follows. 0.5-1.0ml of 1% lignocaine (Xylocaine®; Astra-Zeneca, Kings Langley, UK) was infiltrated into the subcutis under the biopsy sites, which were marked with a circle in indelible pen. 2-4 biopsies were taken from each dog depending on whether lesion skin was present. Biopsies were not taken from dogs where permission was not given. Non-lesional skin biopsies, with no clinical evidence of excoriation or inflammatory skin disease, were taken from the flank. Lesional skin biopsies were taken from areas of erythema and macular-papular dermatitis on the trunk or proximal limbs, with no evident excoriation, staphylococcal pyoderma or *Malassezia* dermatitis (Chapter 2.1.4). The skin biopsies were taken with a six milli-

metre biopsy punch (Stiefel Laboratories, High Wycombe, UK) and placed immediately into RNA-later® (Ambion Inc.; Austin, TX, USA) to prevent degradation of ribonucleic acid (RNA). Samples in RNA-later® were kept at 4°C for 24 hours, then stored at -20°C. Duplicate biopsies from lesional and non-lesional sites were placed in 4% formalin for histopathology (Chapter 2.8). The biopsy sites were closed with a single interrupted polyglactin suture (Vicryl®; Ethicon Inc., Somerville, NJ, USA).

2.2.4 Control tissue samples from healthy dogs

Healthy dogs were sedated and skin biopsies were collected into RNAlater® as previously described (Chapter 2.2.3). Skin biopsies were taken from the euthanasia cases immediately after euthanasia. Popliteal lymph nodes and tonsils were also excised from these dogs to act as positive control material for the amplification of gene transcripts (Chapter 2.7). These tissues were divided into $5mm^3$ portions and stored in RNAlater® as described (Chapter 2.2.3).

2.3 *Dermatophagoides* species house dust mite extracts

Freeze-dried *Dermatophagoides* species mite extracts were obtained from a number of sources (Table 2.2). These were reconstituted in 1ml sterile phosphate buffered saline (80mM Na_2HPO_4, 20mM $NaH_2PO_4.2H_2O$, 100mM NaCl, pH 7.5) (PBS). The protein content of each extract was determined using an enzymatic protein assay according to the manufacturer's instructions (BCA Protein Assay; Pierce Laboratories, Rockford, IL, USA). Each extract was then diluted to 1mg/ml in sterile PBS and stored at -20°C.

Table 2.2 – Source of the *Dermatophagoides* extracts

Greer - Commercially available from Greer Laboratories, Lenoir, NC, USA.

ALK - Kindly provided by ALK-Abéllo, Hørsholm, Denmark.

AP - Kindly provided by Allergo-Pharma, Hamburg, Germany.

IG – Purified from used *Dermatophagoides pteronyssinus* culture media at the Immunobiology Group, MRC Centre for Inflammation Research, University of Edinburgh Medical School, Teviot Place, Edinburgh, EH8 9AG, UK. Kindly provided by Dr. G. Hall.

Allergen extract	**Source**
Crude extracts	
Dermatophagoides farinae	Greer ALK
Dermatophagoides pteronyssinus	Greer ALK AP IG
Dermatophagoides microceras	ALK
Purified extracts	
Der p 1	AP IG
Der f 1	ALK
Der p 2	AP
Der f 2	ALK

2.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.4.1 Buffers and solutions

Resolving buffer	1M Tris-HCl pH8.8
Stacking buffer	0.5M Tris-HCl pH6.8
Tank buffer	25mM Tris-HCl pH8.3 0.2M glycine 1.0% Sodium dodecyl sulphate
Reducing loading buffer	0.125M tris/HCl pH6.8 4% Sodium dodecyl sulphate 20% glycerol 0.005% bromophenol blue 10% 2-mercaptoethanol
Non-reducing loading buffer	0.125M tris/HCl pH6.8 4% Sodium dodecyl sulphate 20% glycerol 0.005% bromophenol blue
Molecular weight markers	Broad range (Biorad Laboratories, Ltd.; Hemel Hempstead, UK)

Solutions	Stacking Gel (4%)	Resolving Gel (10%)
Resolving buffer (ml)	-	2.5
Stacking buffer (ml)	1.4	-
Acrylamide (ml)	0.72	3.35
Deionised water (ml)	2.95	3.05
Sodium dodecyl sulphate (ml)	0.28	1.0
N',N',N',N'-tetraethylethylenediamine (Temed) (µl)	10	10
Ammonium persulphate (µl)	100	100

Coomassie blue staining	
Coomassie blue	0.1% Coomassie brilliant blue 25% methanol 5% acetic acid
Destain 1	25% methanol 7.5% acetic acid
Destain 2	45.5% methanol 9% acetic acid

Silver staining	
Fixer 1	10% acetic acid 50% methanol
Fixer 2	7% acetic acid 5% methanol
Reducer	0.0005% dithiothreitol
Silver solution	0.1% silver nitrate
Developer	3% Na_2CO_3 0.02% formaldehyde

2.4.2. Technique

The gel apparatus was assembled according to the manufacturer's instructions (Mini-Protean II®; Biorad Laboratories Ltd.), using the 4% stacking and 10% resolving gels. 10µl (i.e. 10µg) of the *Dermatophagoides* species extracts were diluted 1:1 in either the reducing or non-reducing loading buffer, heated at 95°C for five minutes and added to the appropriate wells of the stacking gel. 5µl of the molecular weight markers diluted 1:20 in the reducing loading buffer were similarly heated and added to wells two and nine. Wells one and ten were not used, as these outer lanes distort during electrophoresis (A.D. Pemberton, Department of Veterinary Clinical Studies, University of Edinburgh, UK; personal communication). The gel apparatus was dismantled and the gels placed in the electrophoresis tank according to the manufacturer's instructions (Mini-Protean II®), filling the inner compartment with the tank buffer. Enough tank buffer was added to the outer compartment to cover the base of each gel. Electrophoresis was carried out at 200V for 40 minutes, or until the leading edge of the loading dye was within 2mm of the bottom of the gel.

2.4.3 Staining and imaging

2.4.3.1 Coomassie blue

Gels were stained in 2.5% Coomassie Blue for ten minutes, then briefly immersed in destain 1. The gels were then rinsed in destain 2 with frequent changes of solution until the background was clear.

2.4.3.2 Silver staining

Completed gels were bathed in fixer 1 at room temperature for 15 minutes, rinsed twice in fixer 2 for ten minutes and finally washed twice in deionised water for five minutes. The fixed gels were then reduced at room temperature for 15 minutes and washed three times in deionised water for five minutes. Reduced gels were bathed in the silver solution for 20 minutes at room temperature, then briefly rinsed in deionised water before developing. Once fully developed, the reaction was stopped by adding crystalline citric acid monohydrate to effect and rinsing in deionised water.

2.4.3.3 Imaging of stained gels

Coomassie blue or silver stained gels were transilluminated with white light and photographed (The Imager; Appligene Oncor, Chester-le-Street, UK).

2.5 Immunoblotting

2.5.1 Buffers and solutions

Skimmed milk	0% fat dried milk powder, J. Sainsbury plc, Straiton, UK.
Tris-buffered saline (TBS)	20mM tris-HCl 0.5M NaCl pH 7.5
Lower anode buffer	0.3M tris-HCl pH 10.4 20% methanol
Upper anode buffer	25mM tris-HCl pH 10.4 20% methanol
Cathode buffer	25mM tris-HCl pH 9.4 40mM 6-amino-n-hexonic acid 20% methanol
Blocking buffer	5% skimmed milk/TBS
Diluting buffer	1% skimmed milk/TBS
Washing buffer	0.05% tween 20/TBS
[1]Goat polyclonal anti-canine IgE (IgG)	A40-125; Bethyl Laboratories, Montgomery, AL, USA.
[1]Mouse monoclonal anti-canine IgE (IgG1)	D9#31116; Kindly provided by Dr. D.J. Deboer, University of Wisconsin, Madison, WI, USA.
[1]Horseradish peroxidase conjugated mouse monoclonal anti-goat IgG	GT-34; Sigma, Poole, UK
[1]Horseradish peroxidase conjugated bovine monoclonal anti-mouse IgG1	MCA1421; Serotec, Kidlington, UK
Horseradish peroxidase substrate	Luminol (ECL; Amersham-Pharmacia Biotech, Little Chalfont, UK)

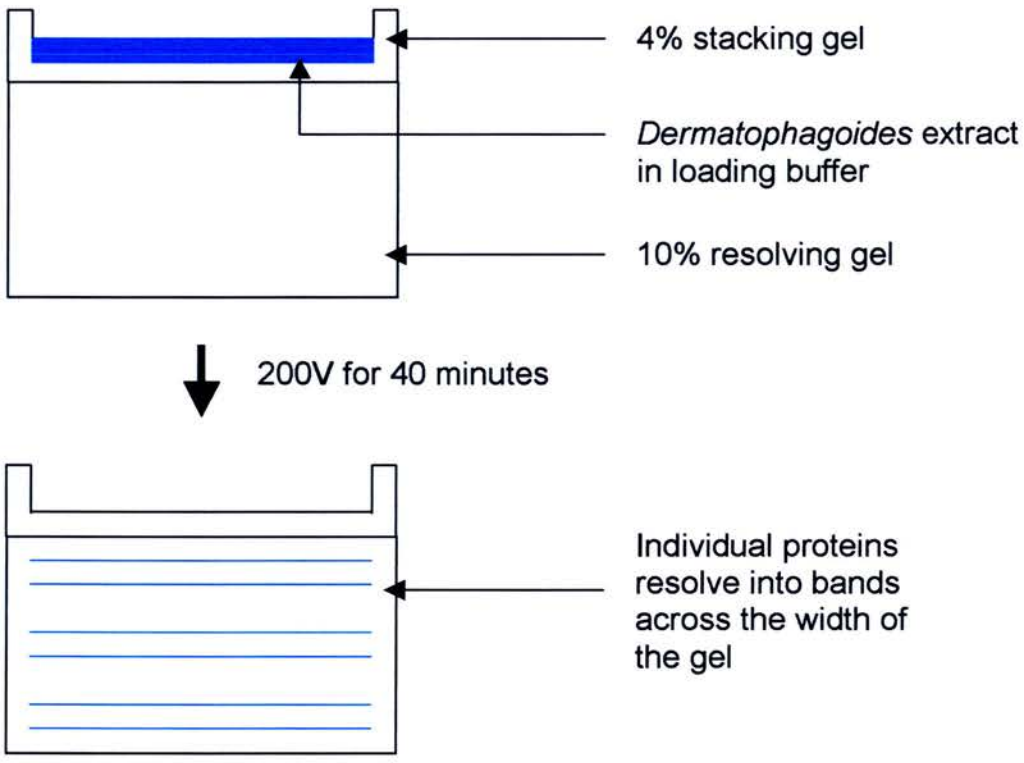
[1]Bethyl Laboratories, Sigma, Serotec and Dr. D.J. DeBoer confirmed the specificity of these reagents (data on file). In addition, they did not bind to western blots of purified canine IgG (T.J. Nuttall and T.-A. Chen; unpublished observations). Preliminary studies also confirmed that there was no non-specific binding to the membranes, allergens, serum or serum heated at 56°C for one hour (see Chapters 2.5.2.2 and 4).

2.5.2 Technique

2.5.2.1 Western blotting

200µl of the crude *D. farinae*, *D. pteronyssinus* and *D. microceras* extracts, and 100µl of the purified group 1 and 2 extracts (i.e. 200µg and 100µg respectively) were diluted as before in reducing and non-reducing loading buffer (Chapter 2.4.2). SDS-PAGE was performed as previously described (Chapter 2.4.2) using a single well to resolve the proteins into bands across the width of the gel (Figure 2.4).

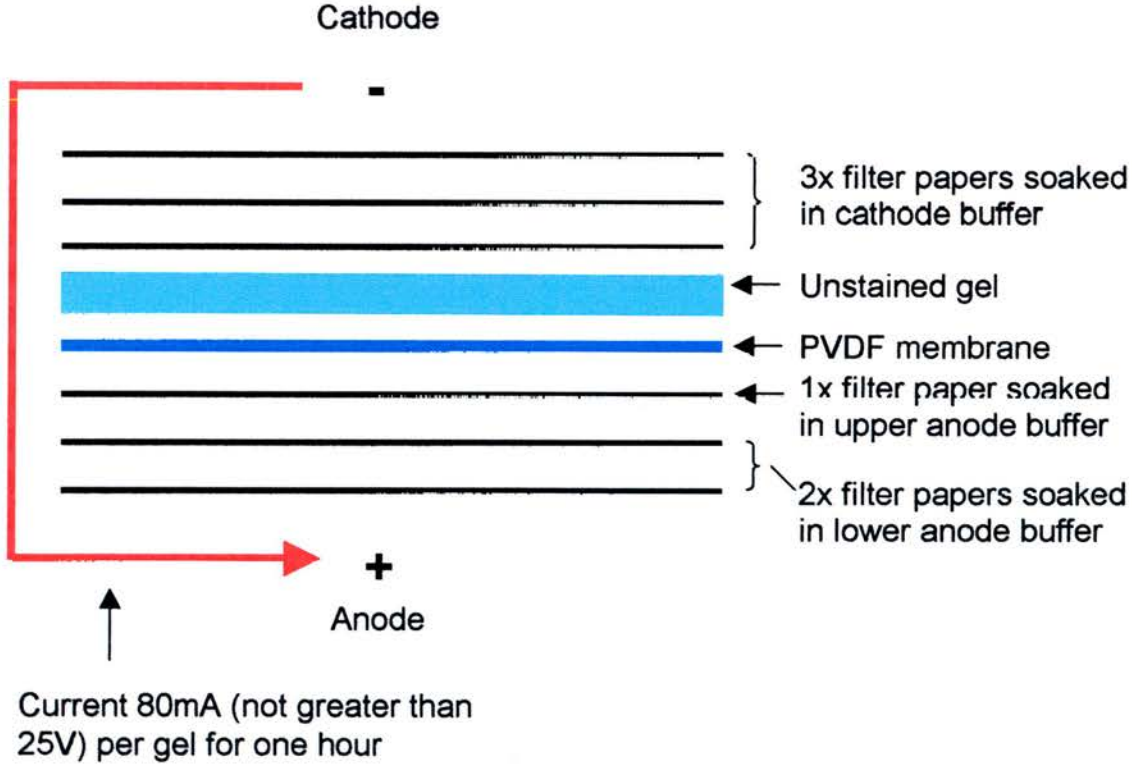
Figure 2.4 – Schematic representation of a single well gel



Proteins from unstained gels were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA, USA). The PVDF membranes

were pre-soaked in methanol for 15 seconds, then rinsed in tris buffered saline (TBS) for two minutes prior to blotting. Two sheets of blotting paper (Whatman no.3; Whatman International Ltd., Maidstone, UK) soaked in the lower anode buffer and a single sheet soaked in the upper anode buffer were laid on the anode surface of the western blot apparatus (Transblot SD®; Biorad Laboratories Ltd.). The PVDF membrane was placed on top of these sheets and the unstained gel placed over the membrane. Three sheets of blotting paper soaked in the cathode buffer were then placed on top of the gel. The apparatus was assembled and run at 80mA (not greater than 25V) per gel according to the manufacturer's instructions (Figure 2.5). After blotting the apparatus was dismantled and the gels stained with Coomassie blue (Chapter 2.4.3) to ensure protein transfer had occurred. The PVDF membranes were air-dried and stored at 4°C.

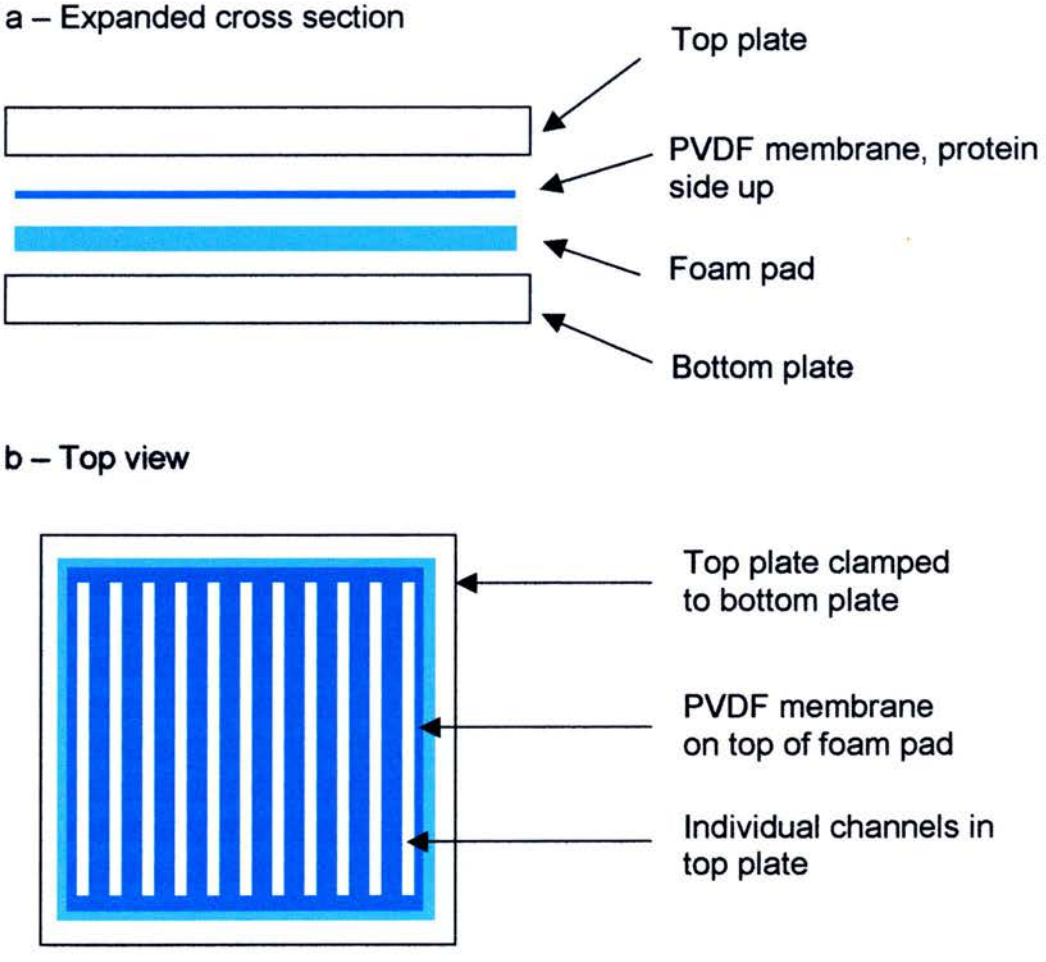
Figure 2.5 – Schematic representation of the western blot apparatus and procedure



2.5.2.2 *Immunoblotting*

The stored PVDF membranes were briefly immersed in methanol then rinsed in TBS for two minutes. The molecular weight markers and a narrow strip to identify protein bands were removed. The remaining portion of the PVDF membrane was incubated with the blocking solution for one hour at room temperature, briefly rinsed in TBS, then placed in an MN28 Miniblotter® (Immunectics, Cambridge, MA, USA), following the manufacturer's instructions. This device exposes narrow strips of the membrane to discrete channels that prevent cross contamination (Figure 2.6).

Figure 2.6 Schematic representation of the MN28 miniblotter®



The appropriate channels were incubated for one hour at room temperature with 85µl of appropriately diluted serum samples, then 85µl of either the mouse monoclonal or the goat polyclonal anti-canine IgE in the dilution buffer. Blanks and control channels with no serum or no anti-canine IgE were included in each assay. Channels were washed with 10ml of the washing buffer after each step. The miniblotter was then dismantled and the membranes incubated at room temperature for one hour with 20ml of the horseradish peroxidase conjugated bovine monoclonal anti-mouse IgG or mouse monoclonal anti-goat IgG in dilution buffer. The membranes were then washed three times for five minutes in the washing buffer and rinsed twice for five minutes in TBS. The optimal serum and reagent dilutions were determined in the series of experiments described in Chapter 4.

2.5.3 Staining and imaging

The molecular weight markers and the protein strip from each membrane were briefly immersed in Coomassie blue stain (Chapter 2.4.3), then rinsed in destain 2 (Chapter 2.4.3) with frequent changes of solution until the backgrounds were clear. The strips were then air-dried.

The protein surface of each probed membrane was covered with the luminol solution for one minute. The luminol was then poured off and each blot placed protein side down on the lightproof image plate of an IS440CF imaging system (Kodak; Rochester, NY, USA). Luminescence was recorded using Kodak 1D software (NEN® Life Science Products, Zaventem, Belgium).

2.6 PBMC isolation and culture

2.6.1 Buffers, solutions and reagents

Phosphate buffered saline (PBS)	80mM Na_2HPO_4 20mM $NaH_2PO_4.2H_2O$ 100mM NaCl pH 7.5
Citrated PBS	PBS 1.2% tri-sodium citrate
Density centrifugation solution	1.007g/ml polysucrose/sodium diatrizoate solution (Histopaque®-1077; Sigma)
Complete medium	Dulbecco's modified Eagle's medium (Sigma) 10% foetal calf serum (FCS) (Serotec, Kidlington, UK) 2mM L-glutamine (Life Technologies, Paisley, UK) 50µM 2-mercapto-ethanol (Life Technologies) 100IU/ml penicillin 100µg/ml streptomycin 0.25µg/ml amphotericin B (100x antibiotic-antimycotic solution; Sigma) 1% non-essential amino acids (Life Technologies)
Canine vaccine antigens	Parvovirus (Parvo C®; Intervet UK Ltd., Milton Keynes, UK) Distempter virus, adenovirus 1 and 2, parvovirus and parainfluenza virus (DHPPi®; Intervet UK Ltd.) *Leptospira* (L®; Intervet UK Ltd.) *Bordetella bronchiseptica* (Intrac®; Schering-Plough Animal Health, Uxbridge, UK)
Mitogens	Concanavalin A (Sigma) Phytohaemaglutinin-P (Sigma) Pokeweed mitogen (Sigma) Lipopolysaccharide (Sigma)
Lysis buffer	Tri-reagent® (Sigma)
Tritiated thymidine	1.0mCi/ml methyl-^{3}H thymidine (CRK120; Amersham-Pharmacia Biotech)

A single batch of FCS, tested for its ability to support cell culture (E.M. Thornton, Department of Veterinary Clinical Studies, University of Edinburgh, UK; personal communication), was used throughout this series of experiments.

2.6.2 Technique

2.6.2.1 PBMC isolation

The optimal conditions for isolation of canine PBMCs were established in the experiments described in Chapter 5. Blood samples from atopic and healthy dogs (Chapter 2.2.2) were diluted 1:1 in citrated PBS. 20ml of each sample was carefully layered over 20ml of the density centrifugation solution in sterile 50ml polypropylene centrifuge tubes (Fisher Scientific). Each tube was then centrifuged at 440g for 20 minutes at 20°C. Cells were collected from the interface, placed in clean sterile 50ml polypropylene centrifuge tubes and washed with a 10x volume of chilled citrated PBS. The tubes were then centrifuged at 250g for ten minutes at 4°C and the supernatant discarded. The cell pellet was re-suspended in 9ml of sterile distilled water for 30 seconds to lyse any remaining erythrocytes, before adding 1ml of 10x PBS. The cell suspension was then washed in a 3x volume of chilled citrated PBS, centrifuged at 200g for ten minutes at 4°C and the supernatant discarded. The cell pellet was re-suspended in 5ml of the complete medium and kept on ice.

2.6.2.2 Cell viability

10µl of the cell suspension in complete medium was diluted 1:1 with trypan blue (Sigma). Viable cells were counted in a modified Neubauer haemacytometer. Cell suspensions with less than 95% viability were discarded. The cell suspension was then adjusted to $1x10^6$ viable cells/ml in complete medium and kept on ice. 100µl of this suspension was centrifuged onto a glass slide at 10g for three minutes (Cytospin3®; Shandon, Pittsburgh, PA, USA). Slides were air-dried and stained with Diff-

Quik®. The cell population was assessed on morphological characteristics and the numbers of lymphocytes, monocytes, neutrophils and eosinophils recorded.

2.6.2.3 *PBMC culture for* in vitro *proliferation studies*

The *Dermatophagoides* allergen extracts (Chapter 2.3), canine vaccine antigens and mitogens were appropriately diluted in complete medium. 100µl of each dilution was added to the appropriate wells of a 96 well U-bottomed tissue culture plate (Nunclon Microwell®; Fisher Scientific). Wells with 100µl of complete medium were included as negative controls. 100µl of the $1x10^6$ cells/ml suspension (i.e. $1x10^5$ cells) was added to each of these wells. Wells with 200µl of complete medium only were included as background. All wells were set up in triplicate. The appropriate dilutions for all the reagents were established in the experiments described in Chapter 5.

Plates were cultured at 38°C in 5% CO_2 for three to seven days. 0.5µCi tritiated thymidine was added to each well for the final six hours of culture. The cells in each well were then captured on a glass-fibre sheet (Microbeta® 1450-472; Wallac, Turku, Finland) using a cell harvester (Harvester 96®; Tomtec, Orange, CA, USA). The sheets were air-dried and sealed in counting bags with 4ml scintillant (Optiscint HiSafe®; Wallac). The counting bags were placed in cassettes (1450-472; Wallac) and loaded into a beta emission counter (Microbeta Plus® 1450; Wallac). The activity of each well was recorded as counts per minute (cpm).

2.6.2.4 In vitro *culture of PBMCs for RNA extraction*

1.0ml of the Greer *D. farinae* extract at 50µg/ml in complete medium or concanavalin A (ConA) at 0.5µg/ml in complete medium were added to the appropriate wells

of a 24-well flat-bottomed tissue culture plate (Nunclon Multidish®; Fisher Scientific). These were the optimum concentrations established by experiments described in Chapters 5 and 6. 1.0ml of the cell suspension in complete medium (i.e. $1x10^6$ cells) was added to each well. The plates were cultured at 38°C in 5% CO_2 for either one or five days before harvesting. At the end of this period, the plates were centrifuged at 170g for five minutes. The supernatant was carefully aspirated and 300µl of the lysis buffer added to each well. The plates were then gently agitated at room temperature for five minutes.

Freshly isolated PBMCs were harvested in a similar way. The cell suspension of $1x10^6$ cells/ml in complete medium was centrifuged in a sterile 50ml polypropylene centrifuge tube at 170g for five minutes and the supernatant aspirated. 300µl of the lysis buffer/$1x10^6$ cells was added and the cells gently agitated at room temperature for five minutes.

The lysed cell suspensions from cultured and freshly isolated PBMCs were collected and stored in 1ml aliquots at -20°C.

2.7 Expression of cytokine gene transcripts

2.7.1 Buffers, solutions and reagents

Digoxigenin labelling kit	3' end labelling kit (Roche Molecular Biochemicals, Lewes, UK)
Stop solution	0.2mg/ml glycogen 0.2mM EDTA
Lysis buffer	Tri-reagent® (Sigma)
Hybridisation buffer	Rapid-Hyb® (Amersham-Pharmacia Biotech)
RNase/DNase free water	Promega Corp., Madison, WI, USA.
DNase I	DNA-free® (Ambion Inc.)
Heparinase	Heparinase I (Sigma)
RNase inhibitor	RNAsin® (Promega Corp.)

3-Morpholinopropanesulfonic acid (MOPS) buffer	20mM MOPS 5mM sodium acetate pH 5.2 1mM EDTA pH 8.0 glacial acetic acid to pH7.0
Northern loading buffer	50% glycerol 0.1mg/ml Bromophenol blue
RNA ladder	0.24 – 9.5kB RNA ladder (Life Technologies)
Reverse transcription	Reverse Transcriptase System (Promega Corp.)
Deoxynucleotide triphosphates	Mixed dUTP, dATP, dCTP, dGTP (Promega Corp.)
Deoxyribonucleic polymerase	*Taq* DNA polymerase (Roche Molecular Biochemicals
Loading buffer	6x gel loading dye (Promega Corp.)
DNA ladder	100bp DNA ladder (Promega Corp.)
Denaturing solution	1.5M NaCl 0.5M NaOH
Neutralising solution	0.5M Tris 1.5M NaCl 1.0mM EDTA
SSC	0.15M NaCl 15mM tri-sodium citrate pH 7.5
Hybridisation wash buffer 1	2x SSC 0.1% tween 20
Hybridisation wash buffer 2	1x SSC 0.1% tween 20
Hybridisation wash buffer 3	0.15M NaCl 0.1M maleic acid 0.1% tween 20 pH 7.5
Blocking solution	Blocking reagent (Roche Molecular Biochemicals)
Anti-digoxigenin antibody	Alkaline phosphatase labelled anti-digoxigenin antibody (Roche Molecular Biochemicals)
Alkaline phosphatase substrate	CDP-Star® (Roche Molecular Biochemicals)
Substrate buffer	100mM tris-HCl 100mM NaCl 50mM $MgCl_2$ pH 9.5

2.7.2 Primer design

2.7.2.1 Forward and reverse primers

Forward and reverse primers (Tables 2.4 and 2.5) were designed from published canine mRNA sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IL-2, IL-4, IL-6, IL-10, IL-12p35, IL-12p40, IFNγ, SCF, *c-Kit*, TNFα and TGFβ1 (Genbank®; National Centre for Biotechnology Information, URL - www.ncbi.nlm.nih.gov/). The optimum reaction conditions were determined in the series of experiments described in Chapter 7.

Table 2.4 National Centre for Biotechnology Information accession numbers for canine cytokine mRNA sequences

mRNA sequence	Accession number
GAPDH	AB038240
IL-2	D30710
IL-4	AF083270
IL-6	U12234
IL-10	U33843
IL-12p35	U49085
IL-12p40	U49100
IFNγ	S41201
TNFα	S74068
TGFβ1	AB042265
SCF	S53329
c-Kit	AF099030

Table 2.5 - Cytokine primers and optimum conditions for polymerase chain reactions

Cytokine	Primer sequence	Annealing temperature (°C)	$MgCl_2$ (mM)	pH	PCR Cycles	Product size (bp)
GAPDH	**Forward** - CCTTCATTGACCTCAACTACAT **Reverse** - CCAAAGTTGTCATGGATGACC **Internal** - CCCTCAAGATTGTCAGCAATGCC	55	2.0	8.6	35	400
IL-4	**Forward** - TAAAGGGTCTCACCTCCCAACTG **Reverse** - TAGAACAGGTCTTGTTTGCCATGC **Internal** - CACCAGCACCTTTGTCCACGGA	55	1.5	9.2	36	317
IL-6	**Forward** - CCACAAGCGCCTTCTCCCTGG **Reverse** - TCACGCACTCATCCTGCGACTG **Internal** - GAATCACTACCGGTCTTGTGGAG	60	1.0	8.3	35	532
IFNγ	**Forward** - TCGGACGGTGGGTCTCTTTTCG **Reverse** - CACTTTGATGAGTTCATTTATCGCC **Internal** - CAGCACCAGTAAGAGGGAGGAC	60	2.5	8.9	35	281
IL-2	**Forward** - CTCACAGTAACCTCAACTCCTGC **Reverse** - TTCTGTAATGGTTGCTGTCTCGTC **Internal** - ACACGCCCAAGAAGGCCACAGA	55	2.5	8.6	35	461
TNFα	**Forward** - ACTCTTCTGCCTGCTGCACTTTGG **Reverse** - GTTGACCTTTGTCTGGTAGGAGACGG **Internal** - CACCCACACCATCAGCCGCTTCGCCG	55	2.5	8.9	35	366
IL-12p35	**Forward** - AGCCTCACCGAGCCCAGGA **Reverse** - TAAGGCACAGGACCGTCATAAAAG **Internal** - TGGAGGCCTGCTTACCACTGGA	60	2.0	8.6	34	293
IL-12p40	**Forward** - GCCCCCGGAGAAATGGTGGTC **Reverse** - TTGTGGCACACGACCTTGGCTG **Internal** - ACCAGACCCACCCACAAACCTG	65	1.5	8.3	34	783

Table 2.5 contd. - Cytokine primers and optimum conditions for polymerase chain reactions

Cytokine	Primer sequence	Annealing temperature (°C)	$MgCl_2$ (mM)	pH	PCR Cycles	Product size (bp)
TGFβ	**Forward** - AGTTAAAAGCGGAGCAGCATGTGG **Reverse** - GATCCTTGCGGAAGTCAATGTAGAGC **Internal** - GTTCAGTTCCAGCCCGCCGAGGT	55	2.5	9.2	35	434
IL-10	**Forward** - GTCCCTGCTGGAGGACTTTAAGA **Reverse** - TGGTCGGCTCTCCTACATCTCG **Internal** - AAGGCGGTGGAGCAGGTGAAGA	55	1.5	8.6	35	443
SCF	**Forward** - AGGGATCTGCGGGAAACGTGTG **Reverse** - TGCTGTCATTCCTAAGGGAGCTG **Internal** - CGGGATGGATGTTTTGCCTAGTC	55	1.0	8.6	35	519
c-Kit	**Forward** - GTCACCGTCTGGAAAACTAGTGG **Reverse** - AACAGTCATGGCCGCATCCGAC **Internal** - AACAAATCCATCCCCACACCCTG	55	1.0	9.2	36	462

The primer sequences were designed using Oligo6® software (Molecular Biology Insights, Cascade, CO, USA) to maximise priming efficiency and optimise annealing temperature, whilst minimising hairpin formation and annealing to self or complementary primers. The primer sequences were screened against known sequences to eliminate any cross-reactive oligonucleotides (BLAST®; National Centre for Biotechnology Information; URL – www.ncbi.nlm.nih.gov/). The primers were generated by Cruachem Ltd. (Glasgow, UK), diluted to 20μmol/ml in RNAse-free water (Promega Corp., Madison, WI, USA) and stored at –20°C.

2.7.2.2 Digoxigenin labelling of internal sequence oligonucleotides

Internal sequence oligonucleotides (Table 2.5) were designed as previously described (Chapter 2.7.2.1). 100pmol of each oligonucleotide in 10μl deionised water was incubated with 4μl tailing buffer (1M potassium cacodylate, 0.125M tris-HCl, 1.25 mg/ml bovine serum albumin, pH 6.6), 4μl 25mM $CoCl_2$, 1μl 1mM digoxigenin-ddUTP solution and 1μl (2.5IU) terminal transferase (3' end labelling kit; Roche Molecular Biochemicals, Lewes, UK) for 15 minutes at 37°C. The reaction was stopped on ice with 2μl of the stop solution. The labelled oligonucleotides were diluted in 10ml hybridisation buffer and stored at -20°C.

2.7.3 RNA extraction

2.7.3.1 Technique

The skin biopsy and positive control (lymph node and tonsil) samples stored in RNA-later® were thawed, thinly sliced and placed in 2.0ml of the lysis buffer. The samples were then homogenised on ice in 30-second cycles until fully dissociated

(Ultra-turrax 125®; Janke and Kunkel IKA, Staufen, Germany). The PBMC suspensions stored in the lysis buffer were thawed and thoroughly mixed.

The homogenised samples were allowed to stand at room temperature for five minutes before thoroughly mixing 1.0ml volumes with 0.2ml of chloroform. The solutions were allowed to stand at room temperature for a further five minutes, then centrifuged at 9500g for 15 minutes. The upper layer was collected into fresh tubes and precipitated with 0.5ml isopropanol at room temperature for 60 minutes. The tubes were then centrifuged at 3500g for ten minutes and the supernatant discarded. The RNA pellet was washed in 75% ethanol, centrifuged at 3500g for five minutes and the supernatant again discarded. The washed RNA pellet was dissolved in 50-100µl RNase/DNase free water at 60°C for ten minutes, then chilled on ice.

Genomic DNA was removed by incubating with 4IU DNAse I per 50µl RNA solution at 37°C for one hour. DNAse I was removed by incubating with the inactivating reagent (DNA-free®; Ambion Inc.) at room temperature for two minutes, then centrifuging at 3500g for two minutes.

The RNA concentration was quantified by ultra-violet absorbance at 260nm (DU650 spectrophotometer; Beckman-Coulter, Fullerton, CA, USA), using the formula:

- RNA ng/µl = OD260nm x 40 x dilution factor

The extraction process was repeated for samples with an OD 260nm:OD 280nm ratio of less than 1.5. Samples with a ratio persistently less than 1.5 were discarded. The RNA solutions were stored at -70°C before use.

2.7.3.2 Gel analysis of extracted RNA (Northern gel)

6µl of each RNA solution was mixed with 12.5µl deionised formamide (Sigma), 2.5µl 10x MOPS buffer and 4µl 37% formaldehyde. The RNA solutions were incubated at 65°C for ten minutes, then chilled on ice and 2.5µl Northern loading buffer and 0.5µl of 5% ethidium bromide added to each solution. The RNA samples were then loaded into the appropriate wells of a 0.4% agarose gel in MOPS buffer and 1.85% formaldehyde. 3µl of the RNA markers were mixed with 2.5µl Northern loading buffer and 0.5µl of 5% ethidium bromide and then placed in lane 1 of each gel. Each gel was run for 60 minutes at 70V according to the manufacturer's instructions (Submarine Agarose Gel Unit HE33; Hoeffer Scientific Instruments UK Ltd., Newcastle-under-Lyme, UK). Completed gels were placed on the image plate of an IS440CF imaging system and viewed under 590nm ultra-violet light. Fluorescence was recorded using Kodak 1D software.

2.7.4 Heparinase treatment

Heparin has been shown to inhibit polymerase chain reactions (Izraeli and others 1991). It was likely that heparin would be present in the skin biopsy and positive control (lymph node and tonsil) samples. Any contamination was therefore eliminated by incubating 4µg RNA with 1µl (40IU) RNAse inhibitor, 4µl of tris-HCl (pH 7.5), 1µl of 4mM $CaCl_2$ and 4µl (4IU) heparinase I in a total volume of 40µl at 25°C for two hours. The reaction was quenched on ice and the heparinased RNA stored at -70°C before use. Heparinase treatment of RNA derived from PBMCs was not performed, as it was highly unlikely that these samples would be contaminated with heparin (McAleese and others 1998).

2.7.5 Reverse transcription

1μg of the heparinased (skin, lymph node and tonsil) or non-heparinased (PBMCs) RNA was incubated with 4μl of 5mM $MgCl_2$, 2μl of 10mM tris-HCl (pH 8.8), 2μl of 1mM mixed deoxynucleotide triphosphates, 0.5μl (20IU) RNAse inhibitor, 0.5μl (15IU) avian myeloma virus reverse transcriptase and 1μl (0.5μg) oligo$(dT)_{15}$ primer (Reverse Transcriptase System; Promega Corp.) in a total volume of 20μl at 42°C for 50 minutes. Incubating at 99°C for five minutes and quenching on ice stopped the reaction. The complementary DNA (cDNA) solution was then diluted to 200μl in RNAse free water. 1μg of heparinased or non-heparinased RNA was diluted to 200μl in RNase free water without reverse transcription as a negative control for each sample. cDNA and negative control preparations were stored at -20°C.

2.7.6 Polymerase chain reactions

Polymerase chain reactions (PCRs) were carried out in 50μl volumes containing 10mM Tris-HCl, 2.5IU *Taq* deoxyribonucleic acid polymerase (Roche Molecular Biochemicals), 200μM deoxynucleotide triphosphates, 200nM of each primer pair and 50ng cDNA. The optimum $MgCl_2$ concentration for each primer pair (Table 2.5) was established in the series of experiments described in Chapter 7.

The reaction conditions consisted of initial denaturing at 94°C for two minutes, then cycles of denaturing at 94°C for 30 seconds, annealing for 30 seconds and elongation at 72°C for one minute, before a final elongation at 72°C for seven minutes (GeneAmp® PCR System 2400; Perkin-Elmer, Cambridge, UK). The optimum number of cycles and annealing temperatures for each primer pair (Table 2.5) were established in the experiments described in Chapter 7.

GAPDH was used as the positive control housekeeping gene. PCRs using GAPDH specific primers were performed for each cDNA and negative control preparation. Samples with no GAPDH signal or a positive GAPDH signal from a negative control preparation were discarded.

2.7.7 Analysis of polymerase chain reaction products (Southern gel)

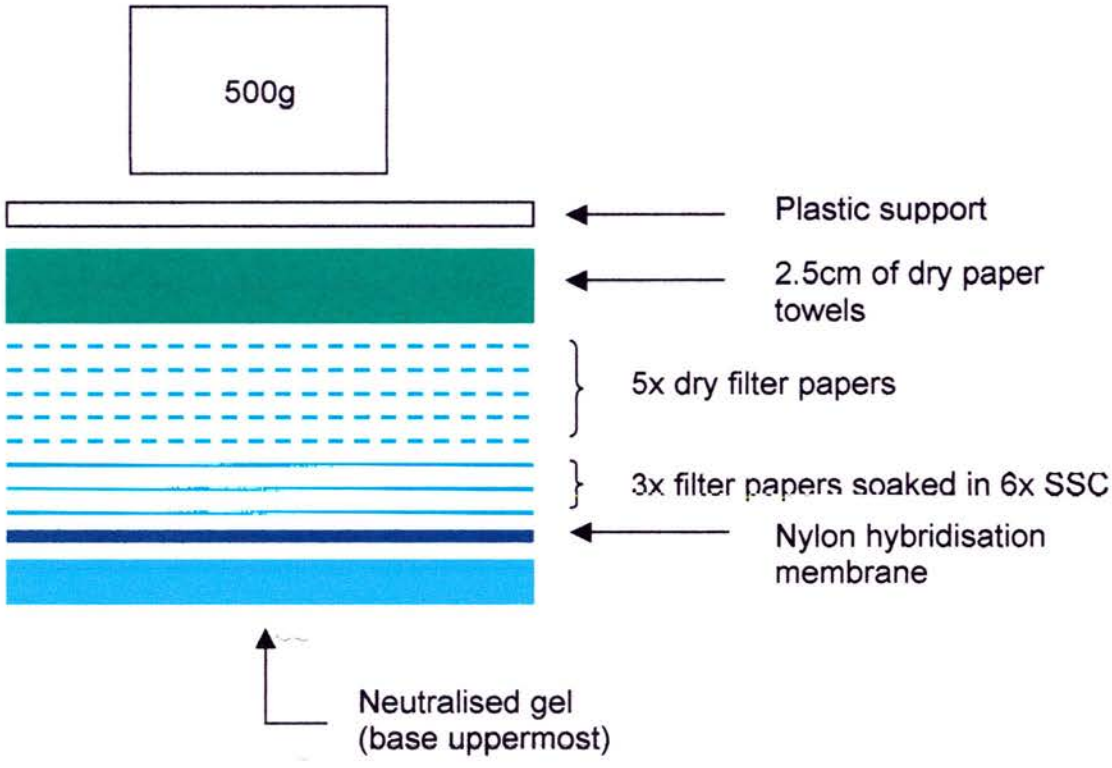
10μl of each PCR reaction was mixed with 2μl of the loading buffer and placed in the appropriate well of a 1.2% agarose gel containing 0.5μg/ml ethidium bromide (Sigma). 2.5μl of the DNA ladder was mixed with 0.5μl of the loading buffer and placed in lane 1 of each gel. Gels were run for 45 minutes at 100V according to the manufacturer's instructions (Horizon II® gel system; Life Technologies). Completed gels were placed on the image plate of an IS440CF imaging system and imaged under 590nm ultra-violet light. The net intensity of fluorescence (net band intensity) of each PCR product was recorded using Kodak 1D software.

2.7.8 Southern blotting and hybridisation

PCRs for each primer pair using positive control cDNA were performed as described previously (Chapter 2.7.6). After imaging the PCR gels were incubated with the denaturing solution at room temperature for 30 minutes. The gels were first rinsed in the neutralising solution for 15 minutes at room temperature, and then for a further 30 minutes in fresh neutralising solution, before a final brief rinse in 6x SSC. The PCR products were then transferred to nylon membranes (Hybond N^{+}®; Amersham International, Amersham, UK) by overnight capillary transfer (Figure 2.7).

After transfer, the hybridisation membranes were rinsed in 6x SSC, air-dried and fixed under 294nm ultra-violet light. They were then incubated in the hybridisation buffer at 40°C for 45 minutes, before incubating for a further three hours at 40°C with the appropriate digoxigenin-labelled internal oligonucleotide sequence in 10ml of the hybridisation buffer. The hybridised membranes were then washed twice for five minutes in wash buffer 1, and twice for 15 minutes in wash buffer 2 at 40°C.

Figure 2.7 – Schematic representation of capillary transfer of PCR products to nylon hybridisation membranes (Southern blot)



The hybridised and washed membranes were blocked at room temperature for 30 minutes, before incubating with the alkaline phosphatase labelled anti-digoxigenin

antibody diluted 1:20,000 in the blocking solution for one hour at room temperature. Membranes were rinsed with wash buffer 3 between each step.

The hybridised membranes were developed by covering the PCR product side with CDP-Star® (Roche diagnostics) diluted 1/1000 in substrate buffer for five minutes. The CDP-Star® solution was then poured off and the membrane placed PCR product side down on the image plate of an IS440CF imaging system. Luminescence was recorded using Kodak 1D software.

2.8 Histopathology and morphometric analysis of the skin biopsy specimens

2.8.1 Processing and staining of the histopathology sections

Skin biopsies of atopic and healthy skin in neutral buffered formalin were embedded in paraffin wax. 4μm sections were oriented parallel to the lie of the hair follicles. After fixing to glass slides, the sections were stained with haematoxylin and eosin (University of Edinburgh Department of Veterinary Pathology, Veterinary Field Station, Easter Bush Veterinary Centre, Easter Bush, Midlothian, EH25 9RG, UK).

2.8.2 Imaging and analysis of the histopathology sections

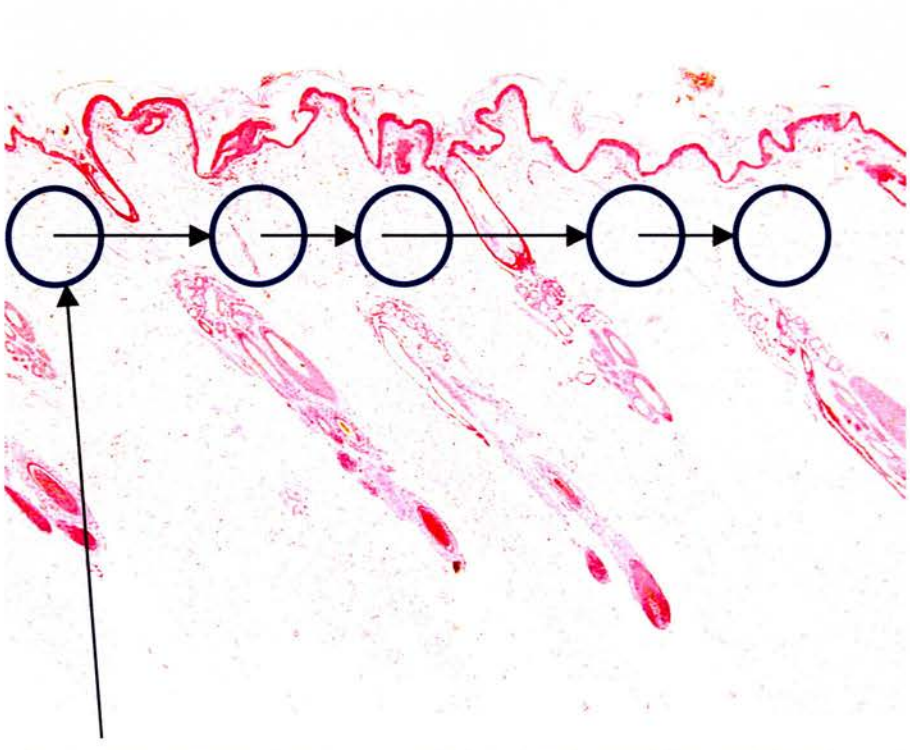
The identity of each section was concealed during subsequent analysis and a single investigator (T.J. Nuttall) assessed all the histopathology sections. Bright field images were acquired with a Sony DXC-390P 3CCD colour video camera (Scion Corporation, Frederick, VI, U.S.A.) mounted on an Axiovert 100 inverted microscope. The RGB video signal from the camera was digitised using Scion Image (Scion Corporation) installed in a G4 Macintosh computer (Apple Computer, Cupertino,

U.S.A.) fitted with a CG-7 RGB 24-bit colour frame grabber (Scion Corporation). Scion Image is a freeware image acquisition and analysis software package, based on NIH Image (URL - www.scioncorp.com).

Five x400 images of the superficial interfollicular dermis were recorded for quantitative analysis. Prior evaluation showed that five images could be obtained from each section by starting at the left edge (epidermis uppermost) and moving across by half a graticule division per image (Figure 2.8). Fields that included adnexal structures were discarded.

Images were prepared for analysis using Object-Image, a public domain software package based on NIH Image developed by Norbert Vischer (The University of Amsterdam, Amsterdam, The Netherlands) (URL – www.simon.bio.uva.nl/ object-image.html). The image software was used to compute the proportion of each image area that consisted of cell nuclei compared to cytoplasm and extracellular matrix.

Figure 2.8 - Diagram illustrating image capture for morphometric analysis of the superficial interfollicular dermis (healthy skin; haematoxylin and eosin stain; x20 magnification)



Five image capture sites in superficial dermis avoiding epidermis and adnexae

2.8.3 Histological descriptions of healthy and atopic skin

After image capture (Chapter 2.8.2) each section was scanned in its entirety at x100 and x400 magnification and the histological findings subjectively described. These assessments were then combined to give a summary of the histopathological features of skin biopsies taken from the healthy and atopic dogs in this study. No analysis was attempted because of the subjective nature of this data.

2.9 Data analysis

Graphical and descriptive statistical analysis of raw data was performed using Prism® (Graphpad Inc., San Diego, CA, USA; URL - www.graphpad.com) and Excel 2000® (Microsoft Corp., Seattle, WA, USA; URL - www.microsoft.com). Excel 2000® was also used to generate standard curves and regression equations. Photodraw 2000® (Microsoft Corp.) was used to illustrate the distribution of clinical lesions.

Data sets with overlapping standard error bars were assumed to show no significant difference (H.Motulsky; The link between error bars and statistical significance, Graphpad Insight, Autumn 2000, URL – www.graphpad.com/articles/errorbars.htm). This minimised the number of tests performed and the chances of a type 1 statistical error (i.e. false positive).

Proportional data were analysed in contingency tables using Fisher's exact tests with significance set at $p<0.05$ (Instat®; Graphpad Inc.). Normality tests were used to assess whether or not continuous data followed a Gaussian distribution (Kolmogorov-Smirnov test with the Dallal and Wilkinson approximation of the p value; Instat®), with the caveat that testing of small samples is only approximate. T-tests with Welch's correction for unequal variance and Mann-Whitney tests were used to compare two sets of Gaussian and non-Gaussian data respectively (Instat®). Paired T-tests and Wilcoxon matched pairs tests were used to analyse matched sets of Gaussian and non-Gaussian data respectively (Instat®). One-way analyses of variance with Tukey's post-tests and Kruskal-Wallis tests with Dunn's post-tests were used to compare three or more Gaussian and non-Gaussian data sets respectively (Instat®). The level of significance in each case was set at $p<0.05$.

3. Clinical and histopathological findings in dogs with atopic dermatitis

3.1 Introduction

Canine atopic dermatitis is essentially a clinical diagnosis. Despite this, the clinical signs have not yet been characterised in detail. Previous studies must be interpreted with caution, as the inclusion criteria often differ or are ill defined (Griffin and DeBoer 2001). Commonly used inclusion criteria include positive intradermal tests or serology. Some dogs, however, fulfil a clinical diagnosis of atopic dermatitis, yet remain persistently negative with these tests.

Colonisation and infection with staphylococcal bacteria and *Malassezia* yeasts are common in both human and canine atopic dermatitis (Rothe and Grant-Kels 1996; Scott and others 2001a). Microbial colonisation correlates with the severity of clinical signs in human atopic dermatitis (Broberg and others 1992; Herz and others 1998). However, although *Malassezia* dermatitis and staphylococcal pyoderma are widely regarded as exacerbating factors in canine atopic dermatitis (Mason 1995; Scott and others 2001a), colonisation has not yet been correlated with the severity of clinical signs.

The aim of this study, therefore, was to evaluate the clinical and histopathological features of allergen reactive and non-reactive dogs with atopic dermatitis. The significance of microbial colonisation was assessed by comparing the clinical severity of lesions in atopic skin with and without secondary infection.

3.2 Materials and methods

3.2.1 Animals and samples

Forty-five dogs with atopic dermatitis and 26 healthy dogs were recruited according to the inclusion criteria described in Chapter 2.1. The age, sex, breed and clinical scores were recorded as described in Chapter 2.1.4 and Figure 2.3. All the dogs with a clinical diagnosis of atopic dermatitis had an intradermal test with 56 allergens (Table 2.1) performed as described in Chapter 2.1.5. Dogs with a clinical diagnosis of atopic dermatitis were divided into those with least one positive intradermal test reaction (n=34) and those with a negative intradermal test (n=11). Dogs with a negative skin test also had negative serology (Chapter 2.1.5). Sera from atopic dogs that had a positive intradermal test were not submitted for an ELISA for financial and clinical reasons.

Skin biopsies of non-lesional (n=23) and lesional (n=13) skin were collected from the atopic dogs as previously described (Chapter 2.2.3). Skin biopsies were similarly collected from the healthy dogs (n=12). These were processed for histopathology as described in Chapter 2.8.1. Morphometric analysis of dermal cell numbers was performed as described in Chapter 2.8.2.

3.2.2 Clinical scores

The mean lesion scores for erythema, papules, scaling, seborrhoea, lichenification and hyperpigmentation (as defined in Chapter 2.1.4) at each body site examined were calculated for the atopic dogs with positive and negative intradermal tests. The presence or absence of pruritus as reported by the owner of each dog was also recorded for each body site (Chapter 2.1.4).

The presence or absence of staphylococcal pyoderma and *Malassezia* dermatitis at each body as determined by the clinician in charge of each case was recorded as described in chapter 2.1.4. To enable valid comparisons only matched body sites that underwent similar clinical and cytological investigation to identify microbial infection were used for statistical analysis.

3.3.3 Data analysis

One-way analyses of variance with Tukey's post-tests were used to analyse the age distribution and Fisher's exact tests used to analyse the number of males and females in the three groups of dogs (Chapter 2.9).

The results at each body site were displayed graphically on a schematic dog outline for the two groups of atopic dogs (Chapter 2.9). No analysis was performed with the healthy dogs, since by definition these animals were lesion-free (Chapter 2.1.2). The percentage of atopic dogs in each group that exhibited pruritus, staphylococcal pyoderma or *Malassezia* dermatitis at each body site were also calculated and similarly displayed. Again, no analysis was performed with the healthy dogs.

The clinical lesion scores at each body site on the intradermal test positive and negative dogs, and clinical lesion scores at each body site with and without staphylococcal pyoderma and *Malassezia* dermatitis were compared using Wilcoxon matched pairs tests (Chapter 2.9).

3.3 Results

3.3.1 Age, sex and breed of the healthy and atopic dogs

The dogs included in this study ranged from 12 – 96 months of age. There was no significant difference in the mean age between healthy dogs, intradermal test positive atopic dogs and intradermal test negative atopic dogs ($p>0.05$) (Table 3.1).

Table 3.1 – Age range (months) of the healthy and atopic dogs

	Healthy dogs (n=26)	Intradermal test positive atopic dogs (n=34)	Intradermal test negative atopic dogs (n=11)
Mean	46.6	38.6	32.7
Standard deviation	20.8	19.5	17.2
Range	18-78	12-96	18-60

More males than females were present in each of the two groups of atopic dogs. In contrast, female dogs outnumbered males in the healthy group (Table 3.2). There were, however, no statistically significant differences between the numbers of male and female dogs in each group ($p>0.05$).

Table 3.2 – The numbers of male and female dogs in the atopic and healthy groups

	Healthy dogs (n=26)	Intradermal test positive atopic dogs (n=34)	Intradermal test negative atopic dogs (n=11)
Male	12	21	6
Female	14	13	5

Twenty-six breeds of dog were included in this study. The atopic dogs were drawn from 22 different breeds, the most common of which were Labradors, followed by German Shepherd Dogs, Jack Russell Terriers, West Highland White Terriers and Dachshunds (Table 3.3). Fewer Labradors and more crossbreds and Springer Spaniels were seen in the healthy group, although the low number of individuals seen of each breed did not permit statistical analysis. There were, however, no major differences in the range of breeds seen in each group. The lack of robust epidemiological data for the reference population precluded any analysis of relative risk.

Table 3.3 – Breeds of dog included in the study

	Intradermal test positive atopic dogs (n=34)	Intradermal test negative atopic dogs (n=11)	Total atopic (n=45)	Healthy dogs (n=26)
Labrador	7	3	10	2
German Shepherd Dog	1	3	4	4
Jack Russell Terrier	3	0	3	2
West Highland White Terrier	2	1	3	1
Dachshund	3	0	3	0
Boxer	2	0	2	1
Border Collie	1	1	2	1
Mastiff	1	1	2	0
Field Spaniel	1	1	2	0
Staffordshire Bull Terrier	2	0	2	0
English Bull Terrier	1	0	1	0
Springer Spaniel	1	0	1	4
Standard Poodle	1	0	1	0
Rottweiler	1	0	1	1
Cocker Spaniel	1	0	1	1
Bearded Collie	1	0	1	0
Border Terrier	1	0	1	0
Dalmatian	1	0	1	0
Shih Tzu	1	0	1	0
Golden Retriever	0	1	1	0
Shar Pei	1	0	1	0
Crossbred	1	0	1	4
Irish Wolfhound	0	0	0	2
St. Bernard	0	0	0	1
Greyhound	0	0	0	1
Doberman	0	0	0	1

3.3.2 Intradermal test results

The intradermal test results are summarised in Table 3.4. Positive reactions were defined as an erythematous wheal with a subjective score of +2 compared to the histamine positive control and saline negative control sites, which were given scores of +4 and 0 respectively (Chapter 2.1.5). Reactions to house dust and forage mites were most common. 75.6% of all the clinically atopic dogs and 100% of the intradermal

test positive atopic dogs had at least one reaction to this group of allergens. 58.8% of the intradermal test positive atopic dogs had reactions only to house dust and forage mites; the remainder also exhibited reactions to other allergens (see below). The most important species were *Dermatophagoides farinae* and *D. pteronyssinus* with positive reactions seen in 94.1 and 82.4% of the intradermal test positive atopic dogs respectively, although reactions to *Tyrophagus putresceantiae*, *Acarus siro* and *Euroglyphus maynei* were also observed. Reactions to a crude house dust extract were common, but none were seen in the absence of responses to other specific allergens.

Reactions to other allergens were much less common. Responses to epithelial and insect allergens were seen in 17.6% and 25.6% of the intradermal test positive dogs respectively. Positive reactions to pollens were less common still, although responses to weed pollens, seen in 23.5% of the intradermal test positive dogs, were more common than reactions to either tree or grass pollens (8.8%). Reactions to one or more mould allergens made up 14.7% of positive intradermal tests.

Table 3.4 - Skin test reactions in dogs with atopic dermatitis

All dogs with a clinical diagnosis of atopic dermatitis were skin tested with 56 allergen extracts. The number of positive reactions for each extract are expressed as percentage of all atopic dogs tested (n=45) and all skin test positive dogs (n=34)

Allergen extract	% of all atopic dogs (n=45)	% of all skin test positive atopic dogs (n=34)
Dust and dust mites	**75.6**	**100.0**
Dermatophagoides farinae	71.1	94.1
Dermatophagoides pteronyssinus	62.2	82.4
Tyrophagus putresceantiae	42.2	55.9
Acarus siro	37.8	50.0
Euroglyphus maynei	26.7	35.3
House dust	71.1	94.1
Epithelia	**13.3**	**17.6**
Cat epithelia	8.9	11.8
Human epithelia	6.7	8.8
Mixed feathers	2.2	2.9
Cotton linters	2.2	2.9
Horse epithelia	0.0	0.0
Sheep epithelia	0.0	0.0
Insects	**20.0**	**26.5**
Blackfly	13.3	17.6
Flea	11.1	14.7
Mixed moths	6.7	8.8
Mosquito	6.7	8.8
American cockroach	2.2	2.9
German cockroach	2.2	2.9
Culicoides midge	0.0	0.0
Tree pollens	**6.7**	**8.8**
Elder	4.4	5.8
Tag alder	4.4	5.8
Elm	4.4	5.8
Box elder	2.2	2.9
Alder	2.2	2.9
Ash	2.2	2.9
American beech	2.2	2.9
White oak	2.2	2.9
Birch	2.2	2.9
Eastern sycamore	2.2	2.9

Table 3.4 continued - Skin test reactions in dogs with atopic dermatitis

Allergen extract	% of all atopic dogs (n=45)	% of all skin test positive atopic dogs (n=34)
White pine	2.2	2.9
White ash	0.0	0.0
Beech	0.0	0.0
American elm	0.0	0.0
Weed pollens	**17.8**	**23.5**
Red clover	13.3	17.6
Lamb's quarter	6.7	8.8
Chrysanthemum spp.	6.7	8.8
Yellow dock	4.4	5.8
Jerusalem oak	4.4	5.8
Common mugwort	4.4	5.8
Brassica spp.	2.2	2.9
English plantain	0.0	0.0
Grass pollens	**6.7**	**8.8**
Couch	6.7	8.8
Red top	4.4	5.8
Kentucky blue	4.4	5.8
Meadow fescue	4.4	5.8
Perennial rye	2.2	2.9
Orchard	2.2	2.9
Sweet vernal	2.2	2.9
Timothy	2.2	2.9
Yorkshire fog	2.2	2.9
Moulds	**11.1**	**14.7**
Aspergillus	6.7	8.8
Botrytis	6.7	8.8
Oat smut	4.4	5.8
Altenaria	2.2	2.9
Penicillium	2.2	2.9
Barley smut	2.2	2.9

3.3.3 Clinical findings in canine atopic dermatitis

3.3.3.1 Pruritus

The percentages of intradermal test positive and negative atopic dogs that exhibited pruritus at each body site are summarised in Tables 3.5 a and b respectively. Pruritus was observed in most body regions apart from the dorsal neck and, in intradermal test negative dogs, the dorsal body. However, it most frequently affected the feet, ears, muzzle and ventral body in both groups of dogs (Figure 3.1 a and b). There were no significant differences in the proportion of intradermal test positive and negative atopic dogs that exhibited pruritus at any body site ($p>0.05$).

Table 3.5a – Percentage of skin test positive atopic dogs with pruritus, *Malassezia* overgrowth or pyoderma at each body site (n=34)

Body sites	Pruritus	*Malassezia*	Pyoderma
Muzzle	68	2	0
Head	24	2	3
Left ear	91	35	9
Right ear	91	35	9
Dorsal neck	0	0	0
Ventral neck	15	9	0
Dorsal body	3	0	3
Left axilla	32	15	12
Right axilla	32	15	12
Left flank	18	0	0
Right flank	18	0	0
Ventral body	59	15	32
Left groin	59	15	41
Right groin	59	15	41
Perineum	15	0	3
Tailhead	3	0	0
***Left fore*:**			
Upper limb	6	3	3
Distal limb	3	3	0
Metacarpals	24	3	0
Dorsal foot	94	26	3
Ventral foot	94	26	6

Table 3.5a continued – Percentage of skin test positive atopic dogs with pruritus, *Malassezia* overgrowth or pyoderma at each body site (n=34)

Body sites	Pruritus	*Malassezia*	Pyoderma
***Right Fore*:**			
Upper limb	6	3	3
Distal limb	3	3	0
Metacarpals	24	3	0
Dorsal foot	94	26	3
Ventral foot	94	26	6
***Left hind*:**			
Upper limb	6	3	6
Distal limb	3	3	0
Metatarsals	24	3	0
Dorsal foot	94	26	3
Ventral foot	94	26	6
***Right hind*:**			
Upper limb	6	3	6
Distal limb	3	3	0
Metatarsals	24	3	0
Dorsal foot	94	26	3
Ventral foot	94	26	6

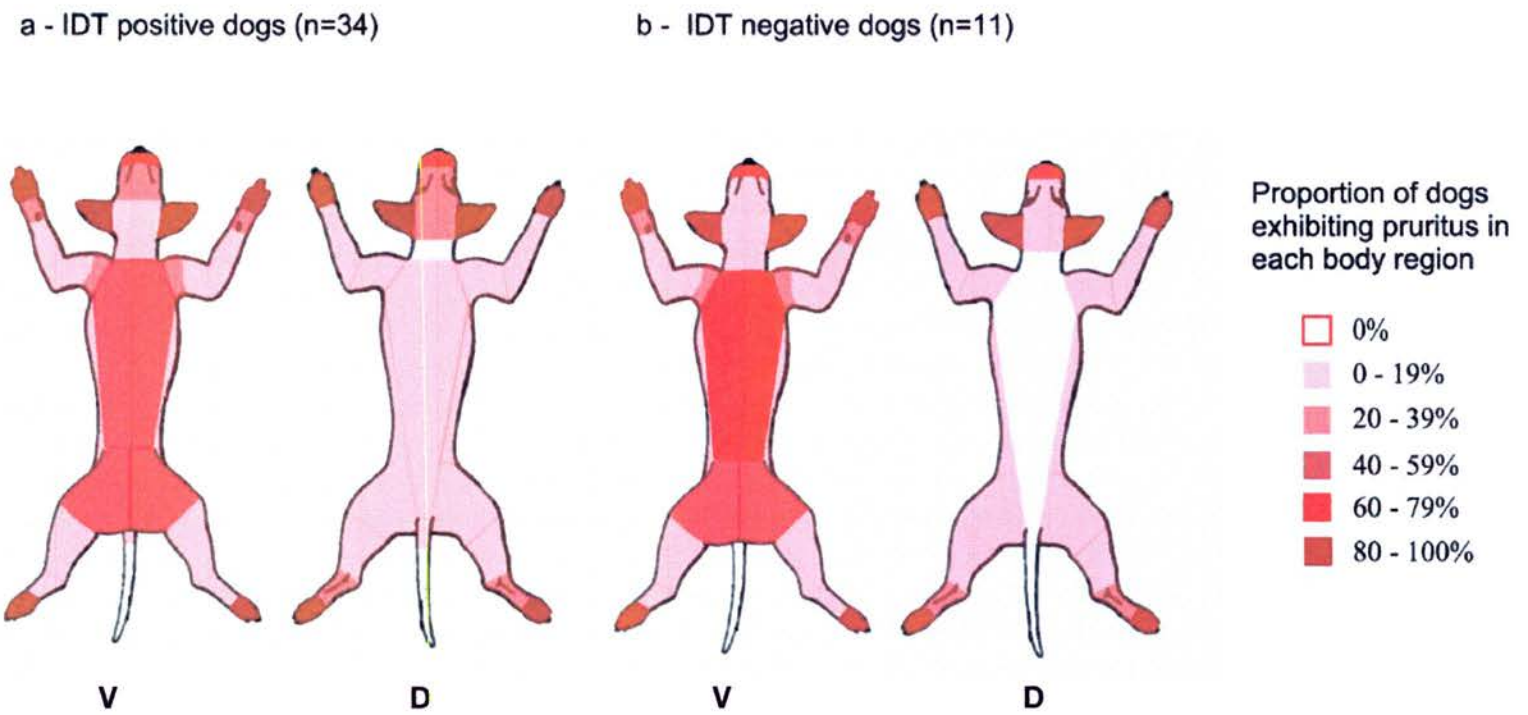
Table 3.5b – Percentage of skin test negative atopic dogs with pruritus, *Malassezia* overgrowth or pyoderma at each body site (n=11)

Body sites	Pruritus	*Malassezia*	Pyoderma
Muzzle	73	0	0
Head	9	0	0
Left ear	91	9	0
Right ear	91	9	0
Dorsal neck	0	0	0
Ventral neck	9	0	9
Dorsal body	0	0	0
Left axilla	36	18	18
Right axilla	36	18	18
Left flank	18	0	9
Right flank	18	0	9
Ventral body	64	18	18
Left groin	55	27	18
Right groin	55	27	18
Perineum	0	0	0
Tailhead	0	0	0
***Left fore*:**			
Upper limb	9	0	9
Distal limb	9	0	0
Metacarpals	36	9	0
Dorsal foot	100	27	0
Ventral foot	91	27	0

Table 3.5b continued – Percentage of skin test negative atopic dogs with pruritus, *Malassezia* overgrowth or pyoderma at each body site (n=11)

Body sites	Pruritus	*Malassezia*	Pyoderma
***Right Fore*:**	0	0	0
Upper limb	9	0	9
Distal limb	9	0	0
Metacarpals	36	9	0
Dorsal foot	100	27	0
Ventral foot	91	27	0
***Left hind*:**			
Upper limb	9	0	9
Distal limb	9	0	0
Metatarsals	36	9	0
Dorsal foot	100	27	0
Ventral foot	91	27	0
***Right hind*:**			
Upper limb	9	0	9
Distal limb	9	0	0
Metatarsals	36	9	0
Dorsal foot	100	27	0
Ventral foot	91	27	0

Figure 3.1 - Distribution of pruritus in dogs with atopic dermatitis



3.3.3.2 *Erythema, papules, seborrhoea, lichenification and hyperpigmentation*

The mean clinical lesion scores for erythema, papules, scaling, seborrhoea, lichenification and hyperpigmentation at each body site in the intradermal test positive and negative atopic dogs are summarised in Tables 3.6a and b respectively.

Erythema was the most prominent clinical sign in both intradermal test positive (Figure 3.2a) and negative (Figure 3.2b) atopic dogs. It was most intense on the ears, feet, metacarpals, metatarsals, axillae, groin, ventral body and muzzle. There was also occasional erythema of the head, trunk and limbs, but with a mean score of less than 1.0 in each case.

Papules, scaling, seborrhoea, lichenification and hyperpigmentation were all much less common than erythema in both intradermal test positive and negative dogs. Papules (Figure 3.3 a and b) were mostly found on the ventral body and less commonly on the limbs and head. Scaling (Figures 3.4 a and b) was an uncommon finding in both groups, but, where seen, was confined to the trunk, head and lower limbs. Seborrhoea was generalised in the intradermal test positive dogs (Figure 3.5a), but largely truncal in intradermal test negative dogs (Figure 3.5b). In both groups, however, the mean clinical score at each body site was less than 1.0. There was slight lichenification of the ventral body and limbs of both groups of dogs, and the head and ears in the intradermal test positive dogs (Figure 3.6 a and b respectively). There was mild hyperpigmentation of the feet, head, ears and ventral body in intradermal test positive dogs (Figure 3.7a). In contrast, hyperpigmentation was more prominent in the groin of the intradermal test negative dogs and absent from the head and ears (Figure 3.7b).

There were no significant differences in the clinical scores for any of these lesions between matched body sites on the intradermal test positive and intradermal test negative atopic dogs ($p>0.05$).

Table 3.6a – Mean lesion scores at each body site in skin test positive atopic dogs (n=34)

Body sites	Erythema	Papules	Scaling	Seborrhoea	Pigmentation	Lichenification
Muzzle	1.4	0.2	0	0.1	0.1	0
Head	0.2	0.2	0.1	0	0.1	0.1
Left ear	3.3	0.5	0.4	0.2	0.5	0.7
Right ear	3.3	0.5	0.4	0.2	0.5	0.7
Dorsal neck	0	0	0.1	0.1	0	0
Ventral neck	0.7	0.3	0.1	0.1	0.1	0.1
Dorsal body	0.1	0	0.8	0.3	0	0
Left axilla	1.3	0.6	0	0.1	0.4	0.3
Right axilla	1.3	0.6	0	0.1	0.4	0.3
Left flank	0.3	0.1	0.7	0.3	0.1	0
Right flank	0.3	0.1	0.7	0.3	0.1	0
Ventral body	1.8	1	0	0.1	0.4	0.4
Left groin	1.9	1.2	0.1	0.1	0.5	0.5
Right groin	1.9	1.2	0.1	0.1	0.5	0.5
Perineum	0.8	0.2	0	0	0	0
Tailhead	0	0	0	0	0	0
***Left fore*:**						
Upper limb	0.1	0.1	0	0.1	0	0.1
Distal limb	0.1	0.1	0	0.1	0	0.1
Metacarpals	1	0.4	0.2	0.1	0.1	0.3
Dorsal foot	2.2	0.1	0.1	0.1	0.1	0.2
Ventral foot	2.3	0.1	0.1	0.1	0.1	0.2

Table 3.6a continued – Mean lesion scores at each body site in skin test positive atopic dogs (n=34)

Body sites	Erythema	Papules	Scaling	Seborrhoea	Pigmentation	Lichenification
***Right Fore*:**						
Upper limb	0.1	0.2	0	0.1	0	0.1
Distal limb	0.1	0.1	0	0.1	0	0.1
Metacarpals	1	0.4	0.2	0.1	0.1	0.3
Dorsal foot	2.2	0.1	0.1	0.1	0.1	0.2
Ventral foot	2.3	0.1	0.1	0.1	0.1	0.2
***Left hind*:**						
Upper limb	0.1	0.2	0	0.1	0	0.1
Distal limb	0.1	0.1	0	0.1	0	0.1
Metatarsals	1	0.4	0.2	0.1	0.1	0.3
Dorsal foot	2.1	0.1	0.1	0.1	0.1	0.2
Ventral foot	2.3	0.1	0.1	0.1	0.1	0.2
***Right hind*:**						
Upper limb	0.1	0.2	0	0.1	0	0.1
Distal limb	0.1	0.1	0	0.1	0	0.1
Metatarsals	1	0.4	0.2	0.1	0.1	0.3
Dorsal foot	2.1	0.1	0.1	0.1	0.1	0.2
Ventral foot	2.3	0.1	0.1	0.1	0.1	0.2

Table 3.6b – Mean lesion scores at each body site in skin test negative atopic dogs (n=11)

Body sites	Erythema	Papules	Scaling	Seborrhoea	Pigmentation	Lichenification
Muzzle	1.5	0.5	0	0	0	0
Head	0.3	0.3	0	0	0	0
Left ear	3	0.3	0	0	0	0
Right ear	3	0.3	0	0	0	0
Dorsal neck	0	0	0.2	0	0	0
Ventral neck	0.1	0	0	0	0	0
Dorsal body	0	0	0.4	0.2	0	0
Left axilla	1.4	0.4	0	0.3	0.6	0.2
Right axilla	1.4	0.4	0	0.3	0.6	0.2
Left flank	0.4	0.2	0.4	0	0.2	0
Right flank	0.4	0.2	0.4	0	0.2	0
Ventral body	2.2	1.1	0	0.3	0.8	0.6
Left groin	2.4	1.2	0	0.3	1.4	0.8
Right groin	2.4	1.2	0	0.3	1.4	0.8
Perineum	0.5	0	0	0	0.4	0.1
Tailhead	0	0	0	0	0	0
***Left fore*:**						
Upper limb	0.3	0	0	0	0	0.2
Distal limb	0.3	0	0	0	0	0.2
Metacarpals	1.4	0.5	0.2	0	0	0.4
Dorsal foot	3.1	0	0	0	0.2	0.3
Ventral foot	3.1	0	0	0	0.2	0.3

Table 3.6b continued – Mean lesion scores at each body site in skin test negative atopic dogs (n=11)

Body sites	Erythema	Papules	Scaling	Seborrhoea	Pigmentation	Lichenification
***Right Fore*:**						
Upper limb	0.3	0	0	0	0	0.2
Distal limb	0.3	0	0	0	0	0.2
Metacarpals	1.4	0.5	0.2	0	0	0.4
Dorsal foot	3.1	0	0	0	0.2	0.3
Ventral foot	3.1	0	0	0	0.2	0.3
***Left hind*:**						
Upper limb	0.3	0	0	0	0	0.2
Distal limb	0.3	0	0	0	0	0.2
Metatarsals	1.4	0.5	0.2	0	0	0.4
Dorsal foot	3.1	0	0	0	0.2	0.3
Ventral foot	3.1	0	0	0	0.2	0.3
***Right hind*:**						
Upper limb	0.3	0	0	0	0	0.2
Distal limb	0.3	0	0	0	0	0.2
Metatarsals	1.4	0.5	0.2	0	0	0.4
Dorsal foot	3.1	0	0	0	0.2	0.3
Ventral foot	3.1	0	0	0	0.2	0.3

Figure 3.2 - Distribution of erythema in dogs with atopic dermatitis

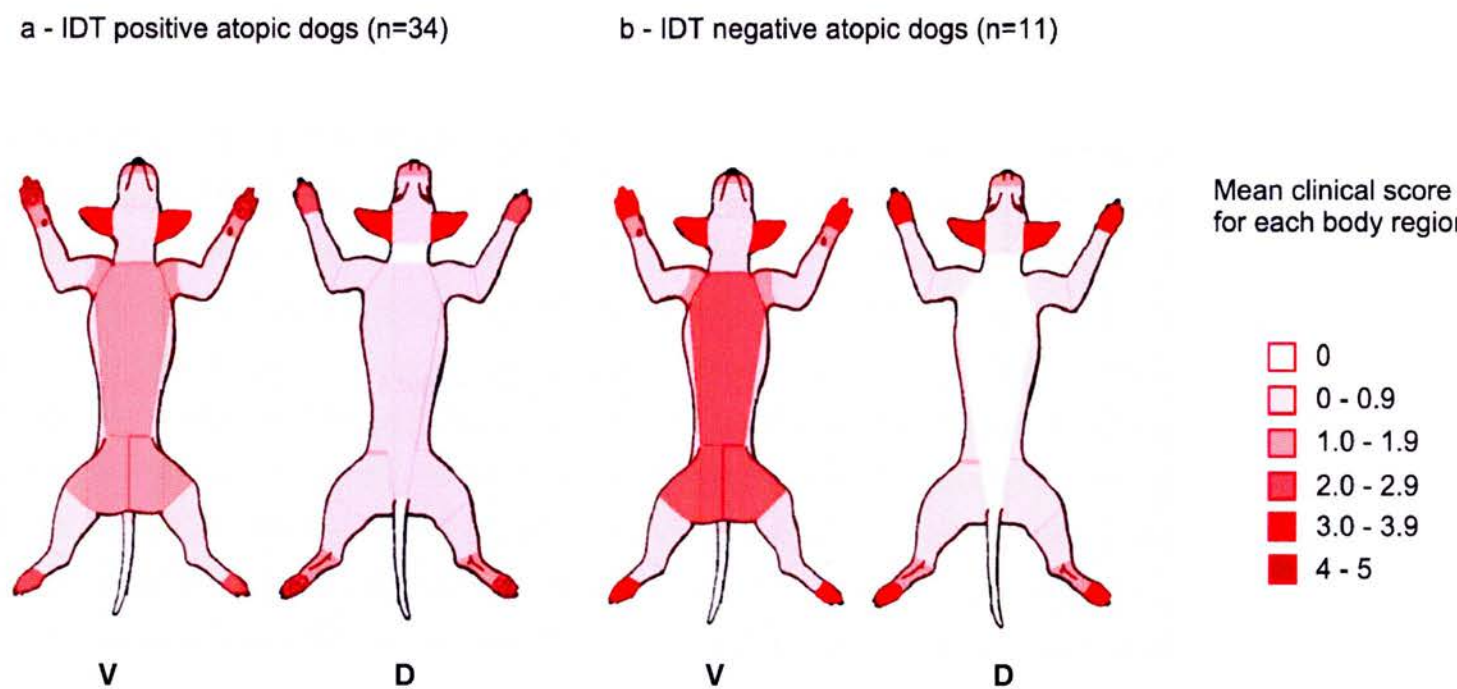


Figure 3.3 - Distribution of papules in dogs with atopic dermatitis

a - IDT positive atopic dogs (n=34)

b - IDT negative atopic dogs (n=11)

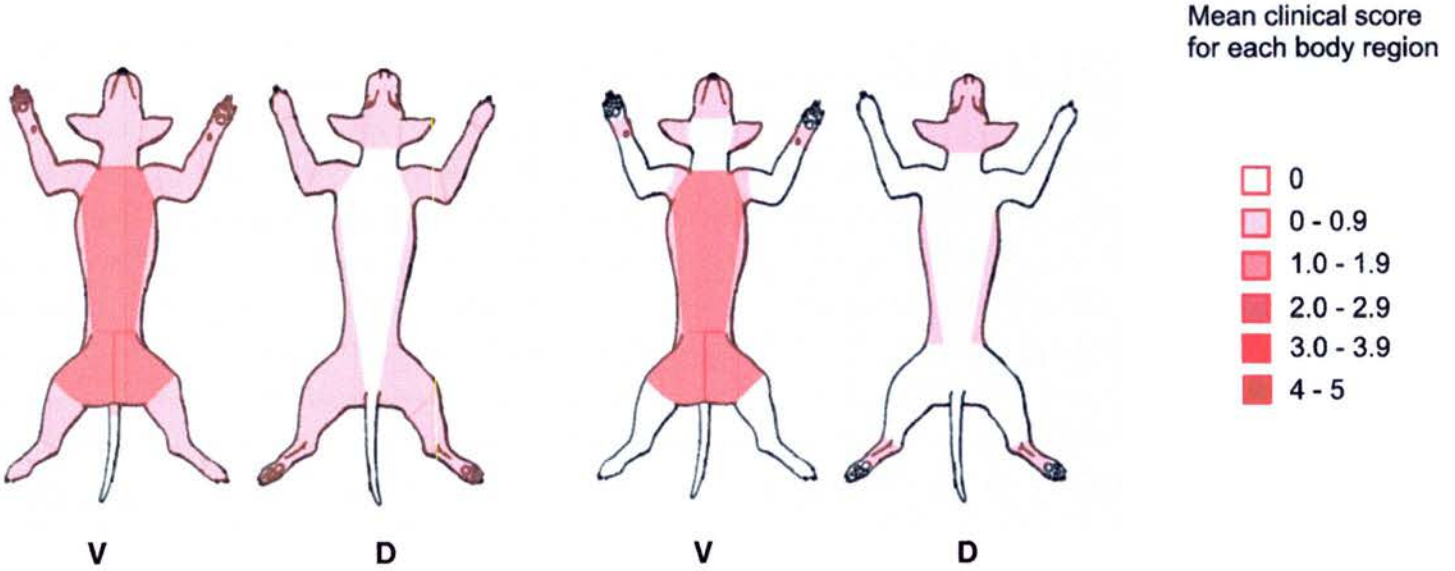


Figure 3.4 - Distribution of scaling in dogs with atopic dermatitis

a - IDT positive atopic dogs (n=34)

b - IDT negative atopic dogs (n=11)

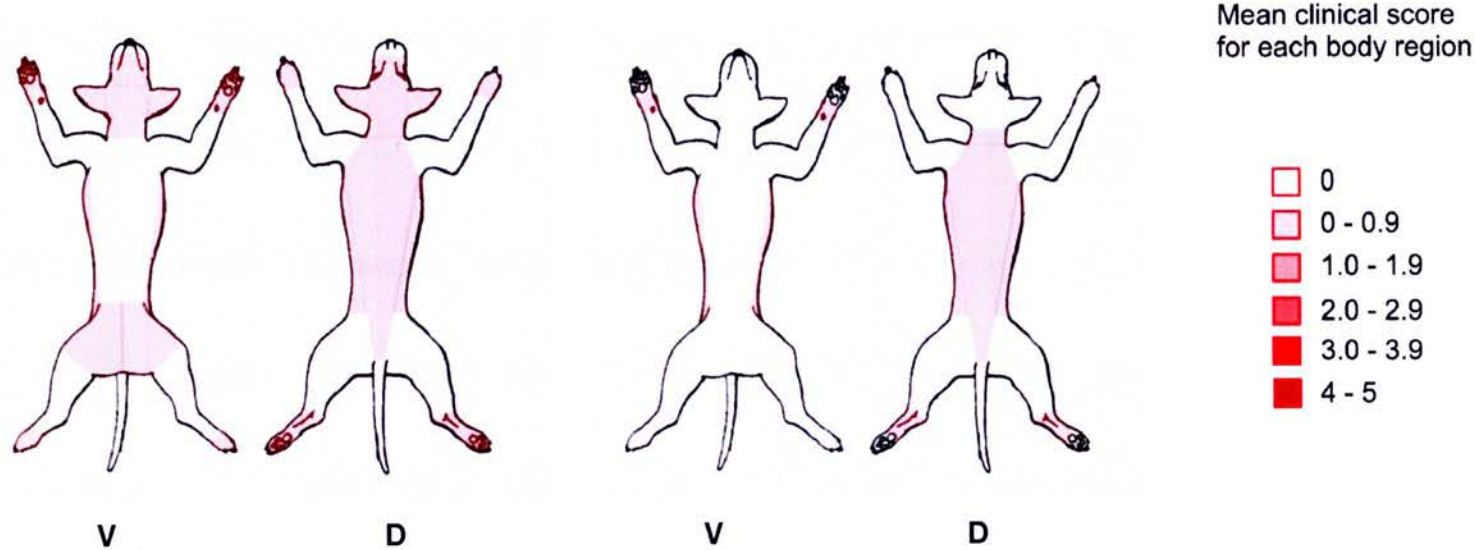


Figure 3.5 - Distribution of seborrhoea in dogs with atopic dermatitis

a - IDT positive atopic dogs (n=34)

b - IDT negative atopic dogs (n=11)

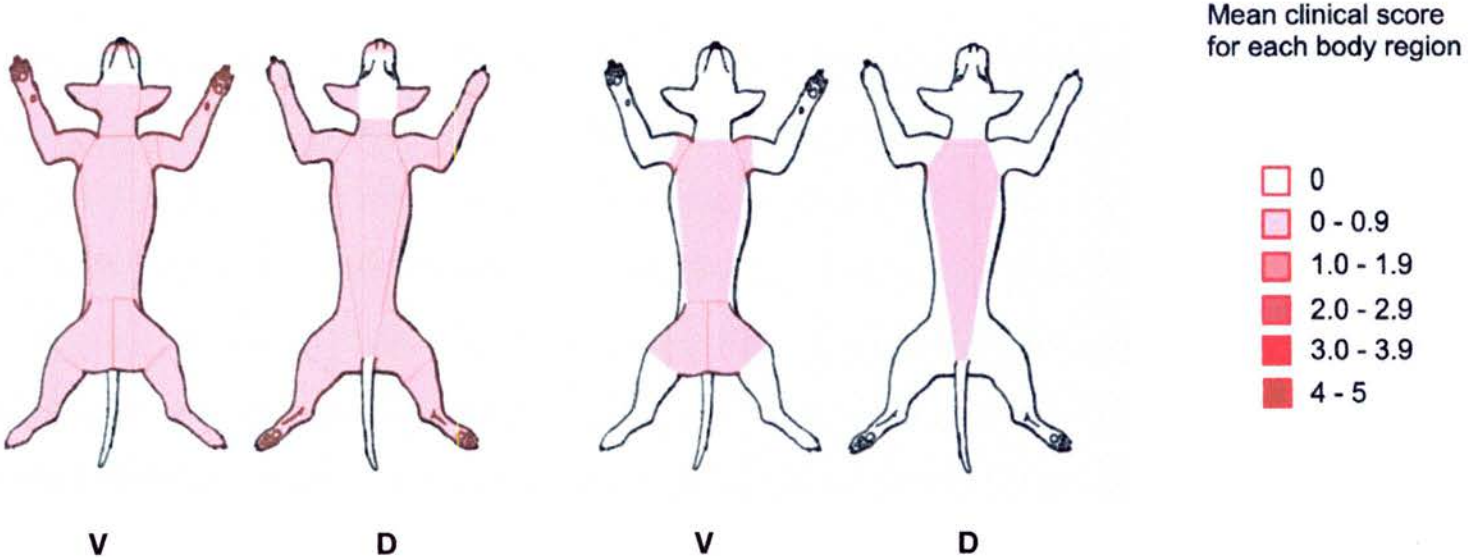


Figure 3.6 - Distribution of lichenification in dogs with atopic dermatitis

a - IDT positive atopic dogs (n=34)

b - IDT negative atopic dogs (n=11)

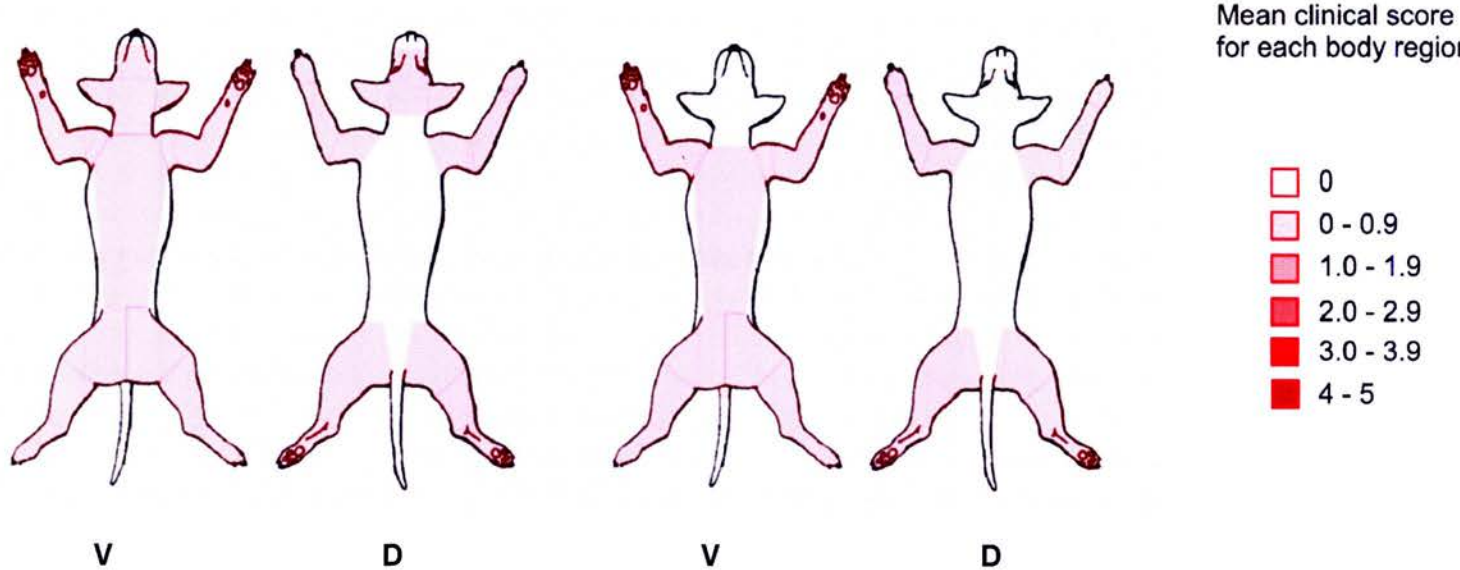
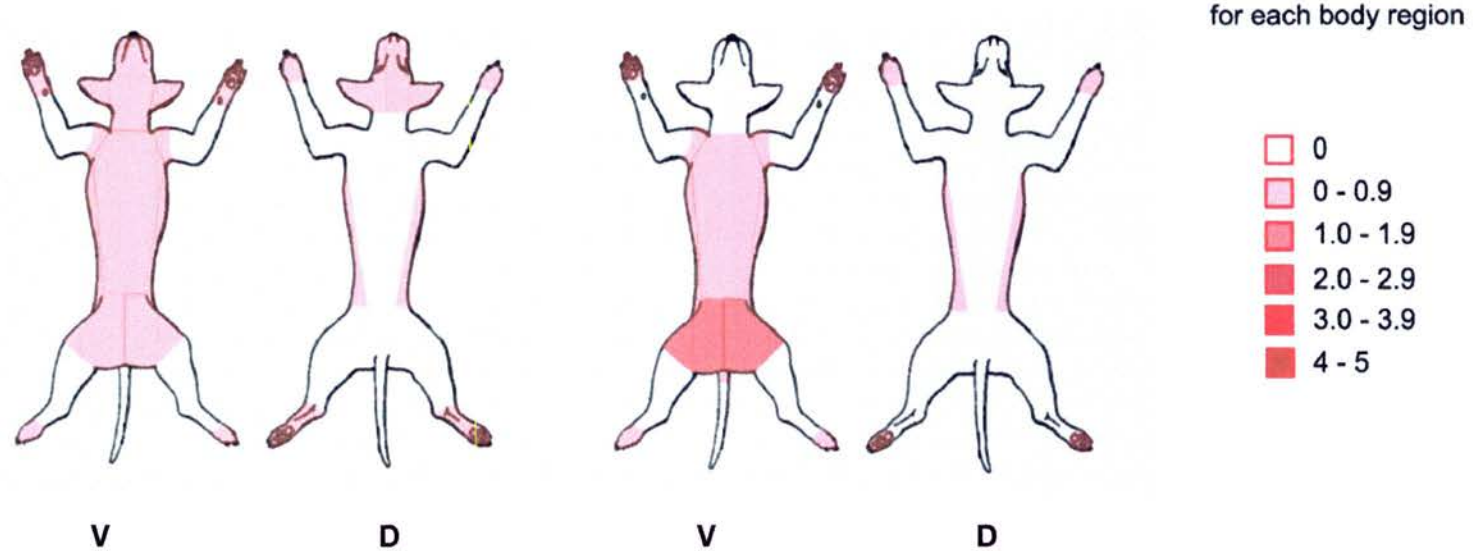


Figure 3.7 - Distribution of hyperpigmentation in dogs with atopic dermatitis

a - IDT positive atopic dogs (n=34)

b - IDT negative atopic dogs (n=11)



3.3.3.3 Malassezia *dermatitis and staphylococcal pyoderma*

The proportions of intradermal test positive and intradermal test negative atopic dogs with clinically significant *Malassezia* or staphylococcal pyoderma at each body site are summarised in Table 3.5 a and b respectively. There was no assessment of the *Malassezia* numbers present in the healthy dogs, although it is likely that the majority of these dogs will have had small commensal populations (Nuttall and Halliwell 2001).

Clinically and cytologically significant *Malassezia* dermatitis was largely restricted to the feet, metacarpals and metatarsals, groin, ventral body and ears (Figure 3.8a and b). However, in the intradermal test positive dogs, *Malassezia* dermatitis was occasionally observed on the limbs and head.

Staphylococcal pyoderma was most frequently seen on the ventral abdomen and groin in both groups of atopic dogs (Figure 3.9a and b). Pyoderma was also infrequently found on the head and ears, back, flanks, upper limbs and feet.

There were no significant differences between the proportion of intradermal test negative and intradermal test positive atopic dogs that exhibited staphylococcal pyoderma or *Malassezia* dermatitis at any body site ($p>0.05$).

Figure 3.8 - Distribution of Malassezia dermatitis and/or otitis in dogs with atopic dermatitis

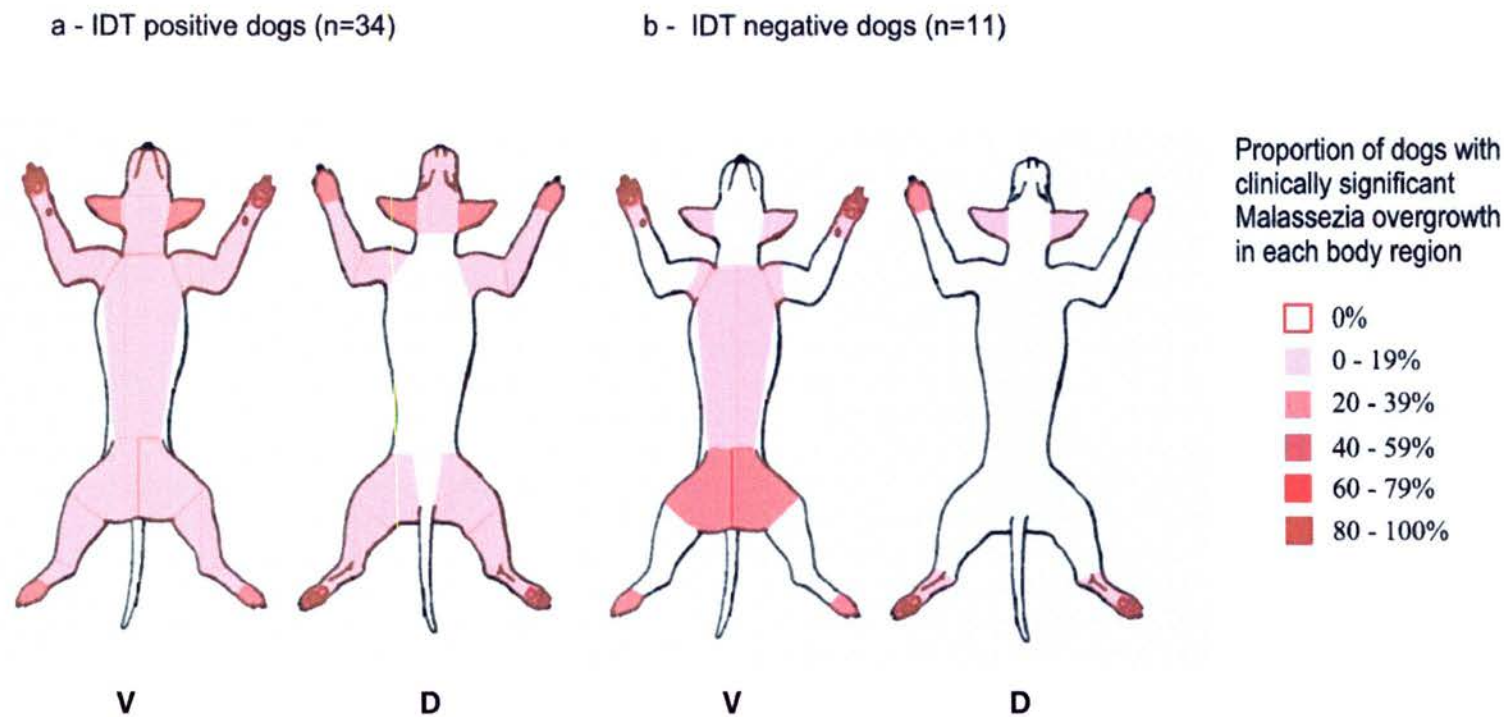
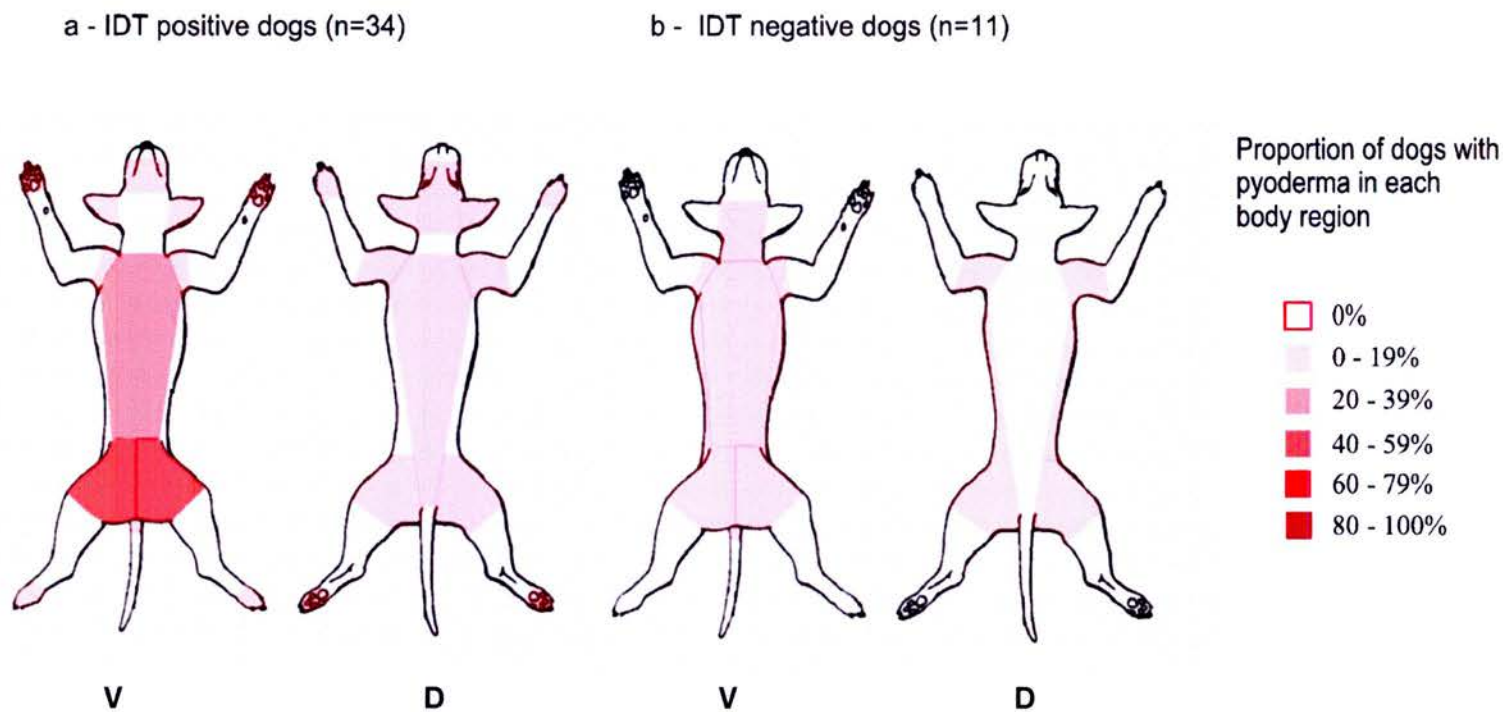


Figure 3.9 - Distribution of staphylococcal pyoderma in dogs with atopic dermatitis



3.3.3.4 *Clinical significance of staphylococcal pyoderma and* Malassezia *dermatitis*

As no significant differences were found in the distribution and frequency of clinical signs and microbial colonisation between the intradermal test positive and intradermal test negative atopic dogs, these data were combined for subsequent analysis.

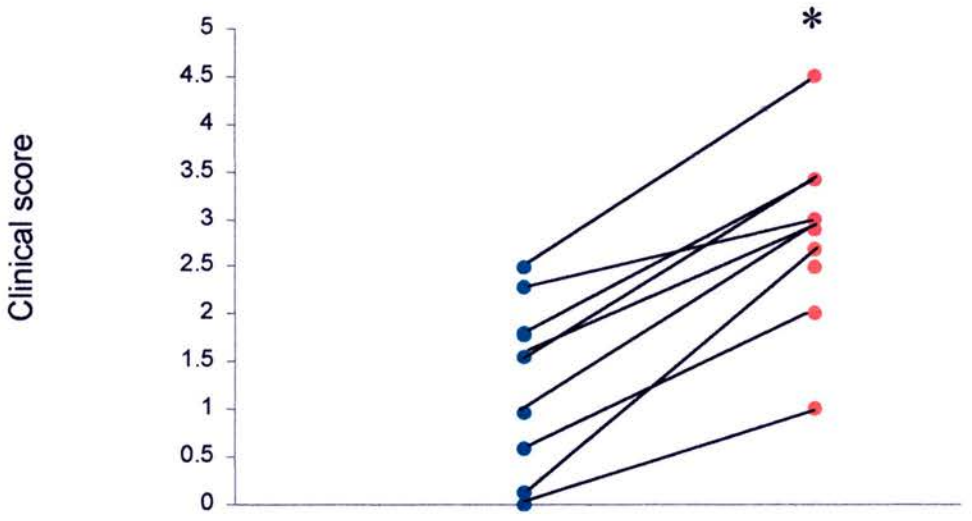
There was a significant increase in mean clinical lesion scores for erythema, scaling, papules, lichenification and hyperpigmentation at body sites with staphylococcal pyoderma compared to matched body sites without staphylococcal pyoderma (Figure 3.10). Lesion scores for seborrhoea at sites with pyoderma were also raised, but in this case there was no significant difference between the two groups.

Mean clinical lesion scores for erythema, papules, seborrhoea and lichenification were also significantly raised at body sites with clinical evidence of *Malassezia* dermatitis compared to matched body sites without *Malassezia* dermatitis (Figure 3.11). Mean lesion scores for scaling and pigmentation were also raised, although not significantly.

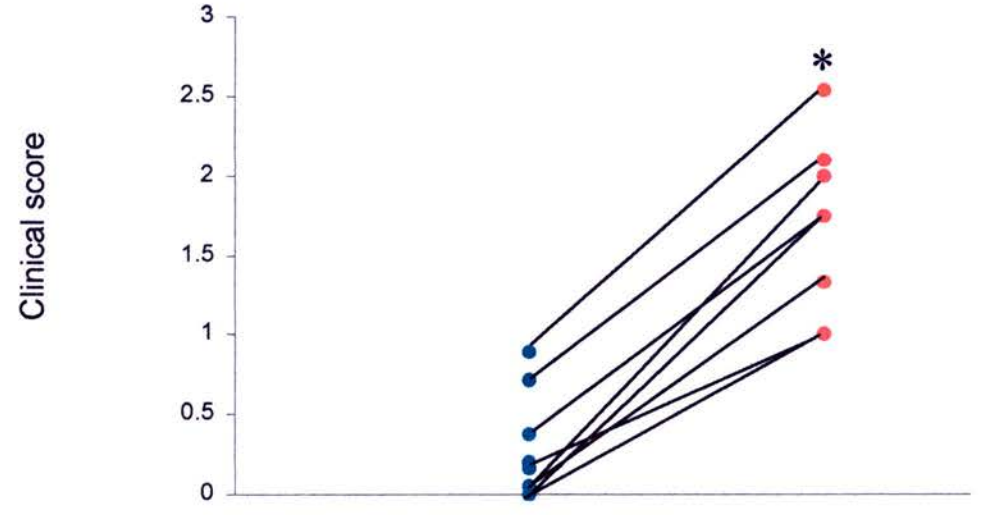
Figure 3.10 – Comparison of clinical scores at body sites with and without staphylococcal pyoderma

● - Body sites without clinical and cytological evidence of staphylococcal pyoderma; ● - body sites with clinical and cytological evidence of staphylococcal pyoderma; * - $p<0.05$, Wilcoxon matched pairs tests

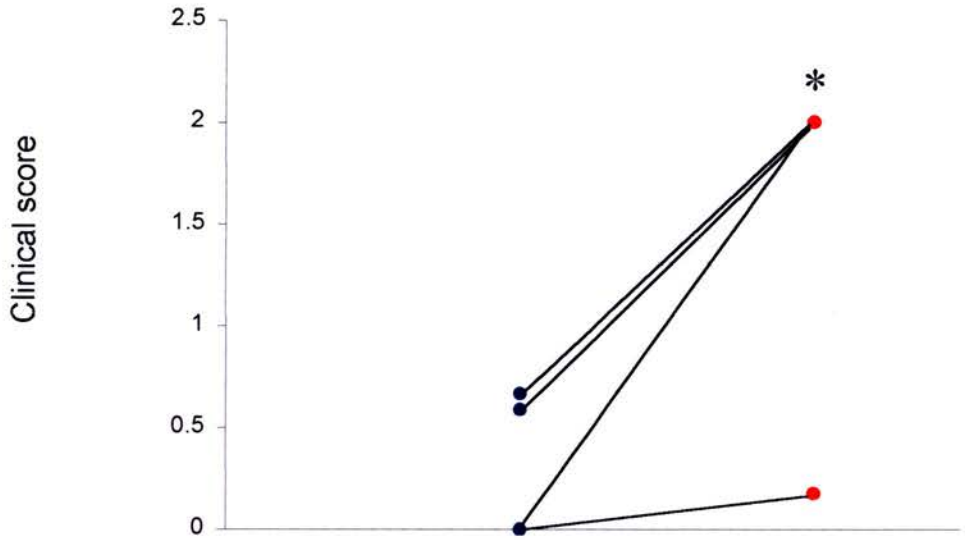
a – Erythema



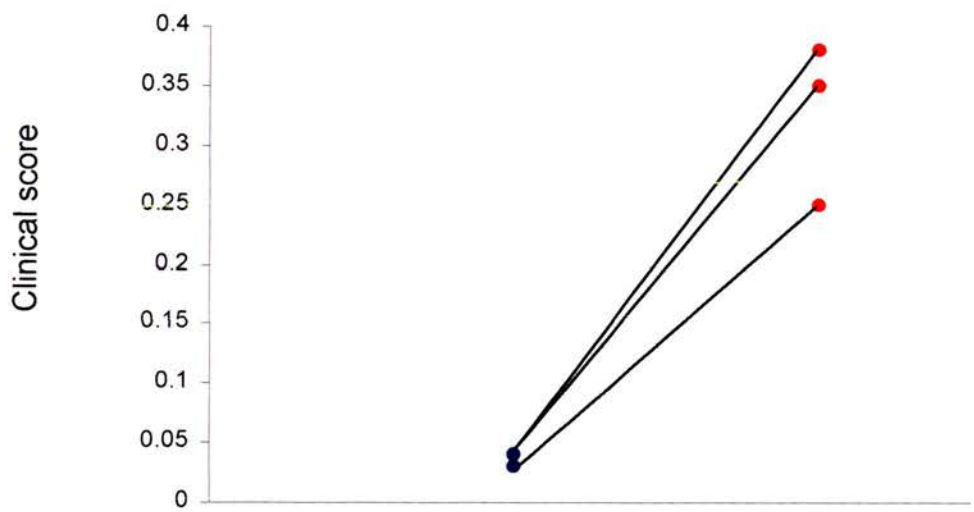
b – Papules



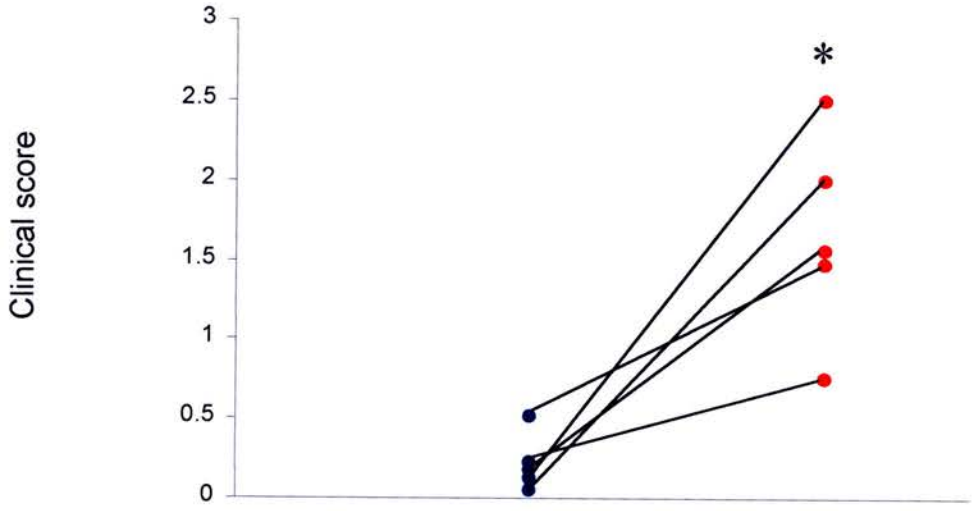
c – Scaling



d – Seborrhoea



e – Pigmentation



f – Lichenification

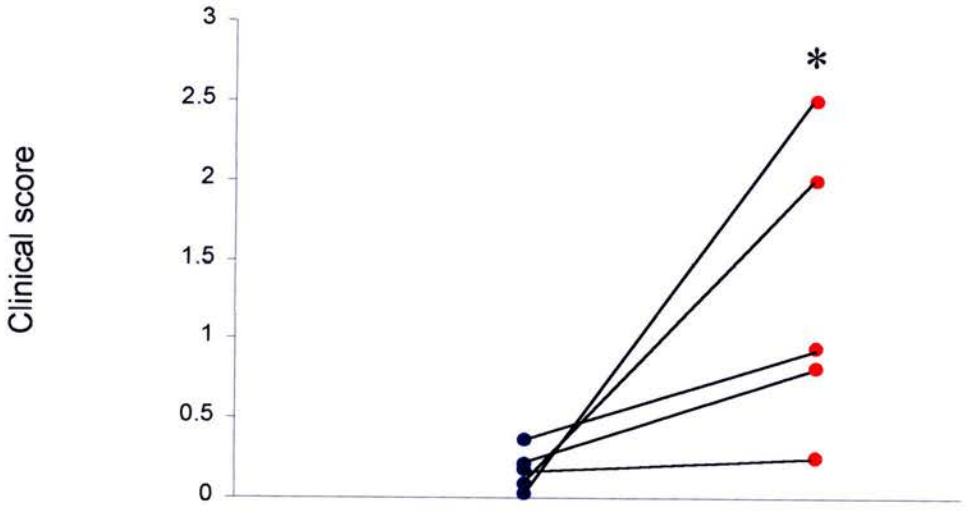


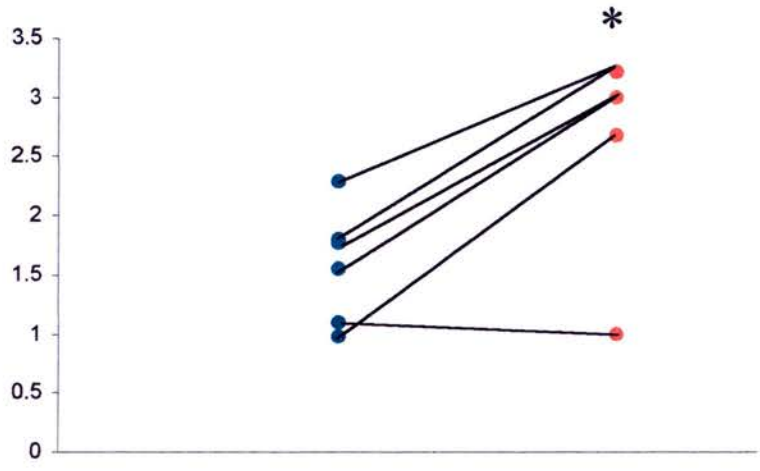
Figure 3.11 – Comparison of clinical scores at body sites with and without *Malassezia* dermatitis

● - Body sites without clinical and cytological evidence of *Malassezia* dermatitis;

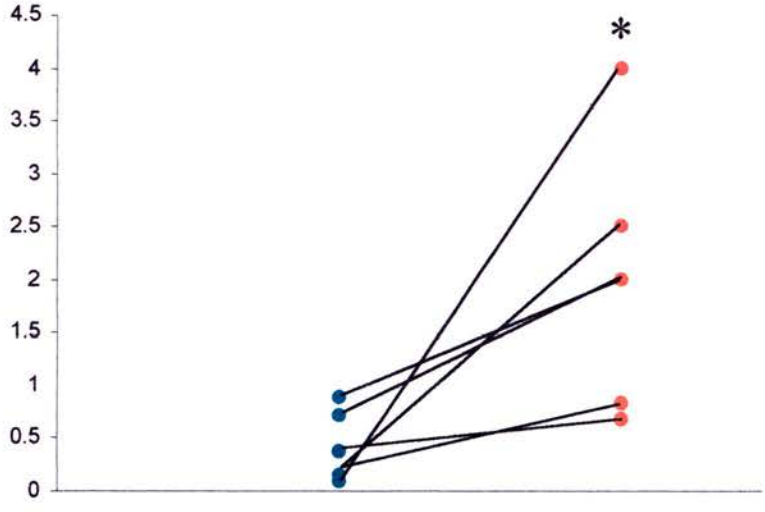
● - body sites with clinical and cytological evidence of *Malassezia* dermatitis;

* - $p<0.05$, Wilcoxon matched pairs tests.

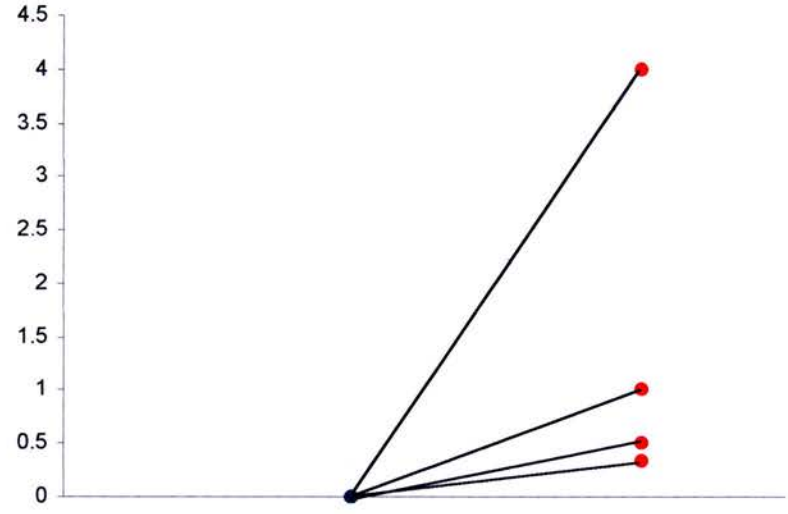
a – Erythema



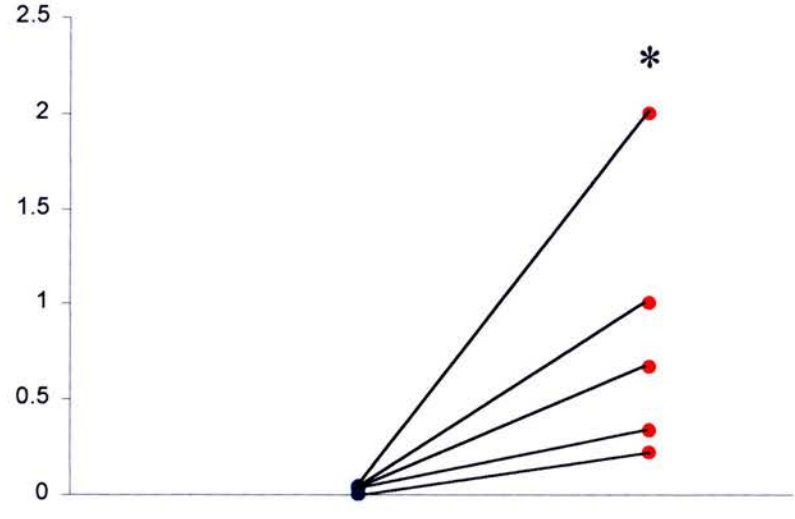
b - Papules



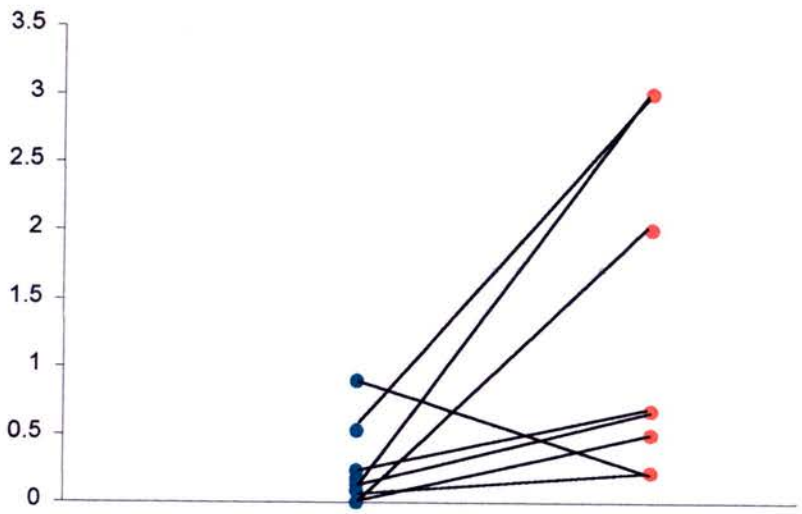
c – Scaling



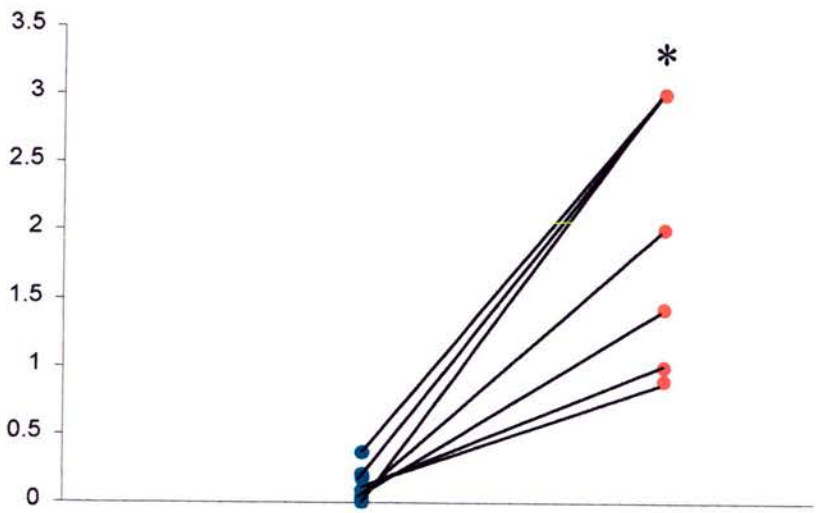
d – Seborrhoea



e – Pigmentation



f – Lichenification



3.3.4 Histopathology of healthy, non-lesional atopic and lesional atopic skin

3.3.4.1 Healthy skin

There were few histopathological findings of note in the biopsies of healthy skin (Figure 12a). There was usually a thin basket-weave stratum corneum overlying an epidermis that was typically 2-3 cells deep. Hair follicles, sebaceous glands, arrector pili muscles, sweat glands and blood vessels were of normal appearance and abundance. The dermis was otherwise occupied by a loose array of fibres with a sparse infiltrate of fibrocytes, mast cells and mononuclear cells.

3.3.4.2 Non-lesional atopic skin

There were mild differences between non-lesional atopic and healthy skin. There was occasional mild basket weave orthokeratotic hyperkeratosis overlying a mildly acanthotic epidermis. Where present, acanthosis and hyperkeratosis was more pronounced in the follicular infundibula. Occasional lymphocyte exocytosis throughout the epidermis was observed in a few samples. Dermal structures were unremarkable, although a mild superficial, perivascular mononuclear cell infiltrate was present in most sections. Mast cells were also frequently seen, but neutrophils and eosinophils were rare (Figure 12b).

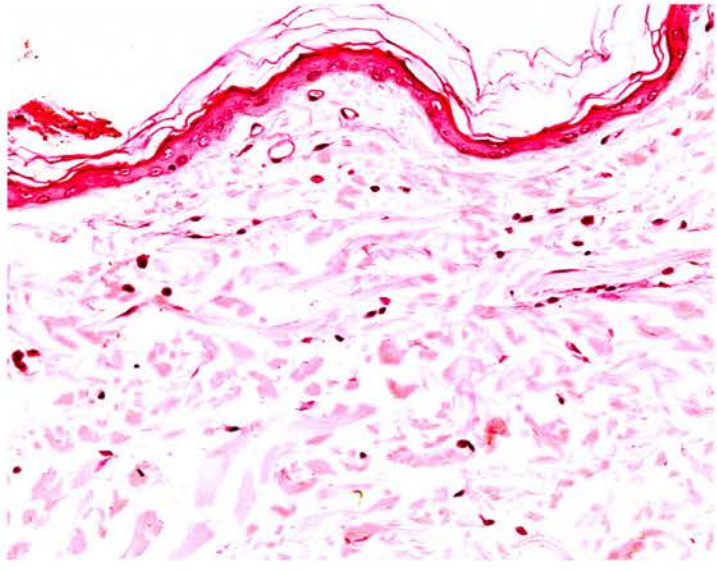
3.3.4.3 Lesional atopic skin

Lesional atopic skin (Figure 12c) was characterised by moderate to severe, basket weave to lamellar orthokeratotic hyperkeratosis, although no microorganisms were observed. There was also moderate to severe acanthosis with focal spongiosis. There was pronounced acanthosis and hyperkeratosis of the follicular infundibula, which

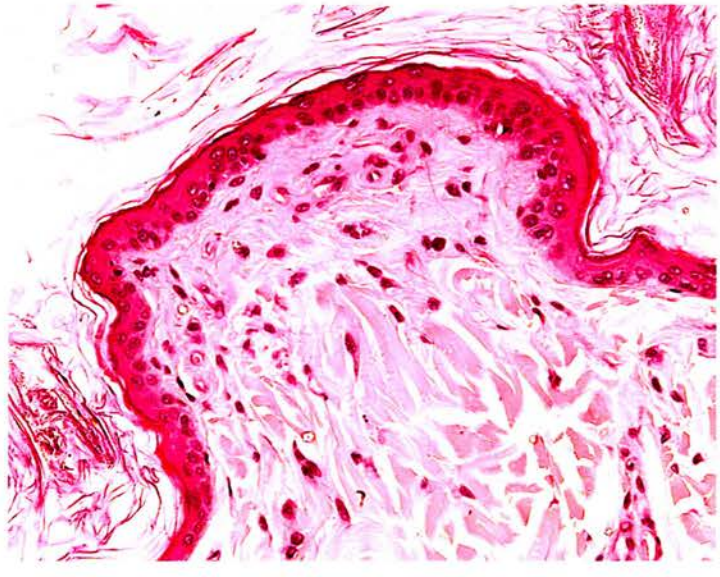
occasionally plugged the follicular ostia. Pseudo-rete peg formation was seen in a few sections. Lymphocyte exocytosis was frequently observed, occasionally forming clusters within the epidermis. Increased amounts of sebaceous tissue compared to healthy skin were noted in a few sections, but otherwise the dermal structures appeared largely normal. There was, however, a moderate to marked cellular infiltrate in all the sections. These were largely superficial, ranging from perivascular to interstitial. The cellular infiltrate largely consisted of mononuclear cells and mast cells, but moderate to large numbers of eosinophils and neutrophils were present in several sections. Focal areas of subepidermal dermal fibrosis were occasionally observed.

Figure 12 - Representative histopathological sections of healthy, non-lesional atopic and lesional atopic skin (haematoxylin and eosin stain; x200 magnification)

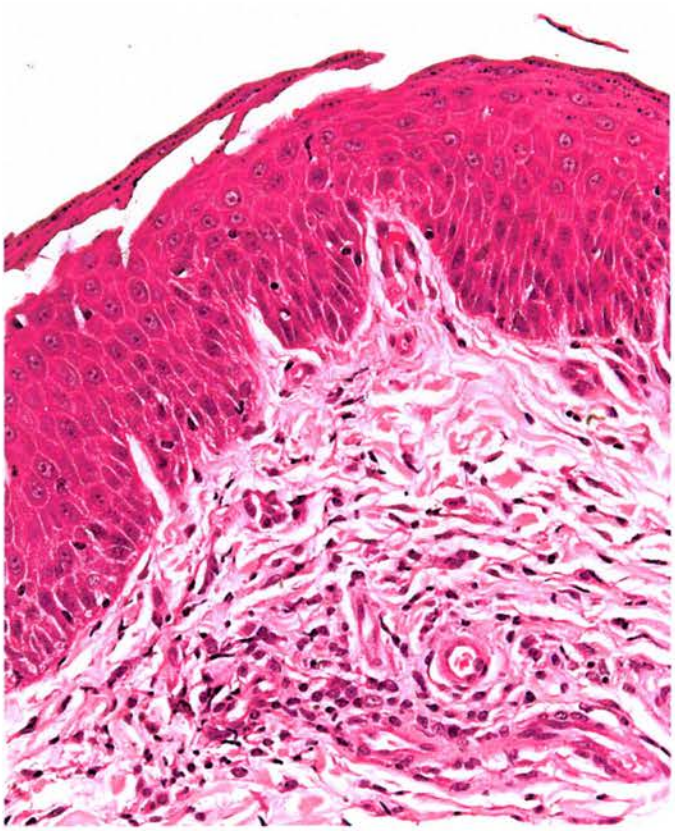
a - Healthy skin



b - Non-lesional atopic skin



c - Lesional atopic skin

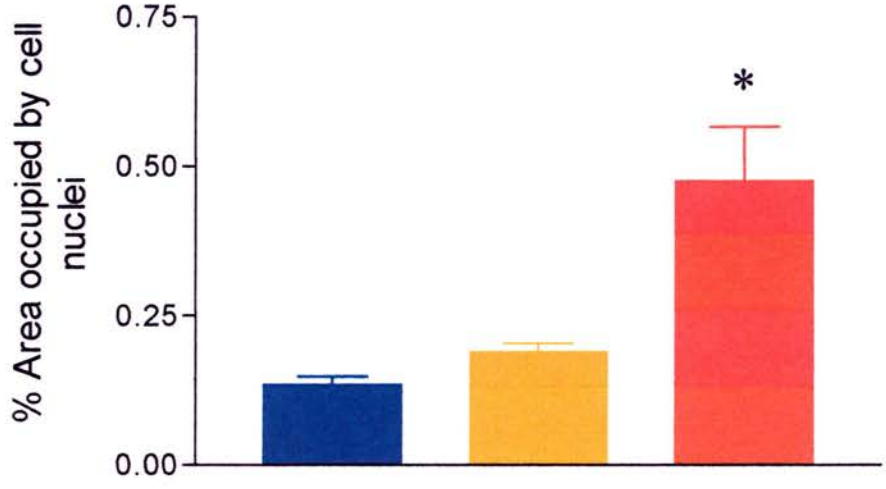


3.3.4.4 *Morphometric analysis of dermal cell numbers*

No histopathological or morphometric differences were noted between non-lesional or lesional atopic skin from intradermal test positive and intradermal test negative atopic dogs. Data from the two groups were, therefore, combined for subsequent analysis. Morphometric analysis revealed that there were significantly greater numbers of cells present in the dermis of lesional atopic skin than in either non-lesional or healthy skin ($p<0.05$). There were more cells present in the dermis of non-lesional atopic compared to healthy skin, but the differences between these two groups were not statistically significant (Figure 3.13).

Figure 3.13 – Morphometric analysis of dermal cell numbers in healthy, non-lesional atopic and lesional atopic skin

■- Healthy skin sections (n=12); ■ - Non-lesional atopic skin sections (n=23); ■ - Lesional atopic skin sections (n=13); bar – sem; * - $p<0.05$



3.4 Discussion

These results characterise in detail the clinical and histopathological findings in the atopic and healthy dogs used in this series of studies. The aim was to ensure that comparisons made between the three groups of dogs and conclusions drawn in later experiments were valid. However, the findings also shed some light on the pathogenesis of canine atopic dermatitis.

3.4.1 The clinical signs of canine atopic dermatitis

The most prominent clinical signs in this study were pruritus and erythema. The other clinical signs, papules, scaling, seborrhoea, lichenification and hyperpigmentation, were much less frequent. These findings support the textbook adage that 'atopic dermatitis is an itch that rashes rather than a rash that itches' (Mason 1995; Scott and others 2001a). A recent review also concluded that the primary lesions largely consist of erythema (Griffin and DeBoer 2001).

Pruritus is also a hallmark of human atopic dermatitis (Rothe and Grant-Kels 1996). It is triggered by many mediators including histamine, serotonin, trypsin, chymase, bradykinin, substance P and vasoactive intestinal peptide (Koblenzer 1999). Pruritus is deeply unpleasant and patients will often self-traumatise to the point where it is replaced with pain (Koblenzer 1999). Human atopic patients may also have erythema or a mild papular eruption, but other lesions are usually secondary to self-trauma and microbial infection (Beltrani 1999a).

The distribution of clinical signs was similar to that widely accepted as compatible with canine atopic dermatitis (Willemse 1986; Mason 1995; Prelaud and others 1998; Griffin and DeBoer 2001; Scott and others 2001a). The most commonly affected

sites, the muzzle, ears, interdigital skin, palmar metacarpals, plantar metatarsals, axillae, ventral body and groin largely correspond to analogous sites affected in human atopic dermatitis (Beltrani 1999a). However, the clinical picture is less close to infantile human atopic dermatitis, where the face and scalp is predominantly involved (Rothe and Grant-Kels 1996; Beltrani 1999a).

The clinical appearance of intradermal test positive and intradermal test negative dogs with atopic dermatitis was very similar. Sensitisation is not necessarily pathognomic for atopic dermatitis, as healthy animals can have positive intradermal tests and elevated IgE titres without clinical signs (DeBoer and Hillier 2001; Scott and others 2001a). However, these results do suggest that there may be two clinically indistinguishable syndromes of atopic dermatitis in dogs, as postulated in humans (Halbert and others 1995). It is unlikely that these dogs represent false negative results as the tests were performed by experienced dermatologists who monitored allergen quality and inhibition by previous therapy. Furthermore, all the dogs with negative intradermal tests also had allergen specific IgE titres beneath the accepted threshold for sensitisation established for a commercial ELISA incorporating recombinant canine FcεRIα (Heska Allercept® test provided by Axiom Laboratories, Teignmouth, UK). In addition, all the atopic dogs had perennial disease and were tested whilst they exhibited clinical signs, making it unlikely that seasonal fluctuations in IgE levels were responsible for negative test results.

This group of dogs may, therefore, represent a subset of patients with non-specific cutaneous hyper-reactivity. There are also cases of human atopic dermatitis that fulfil the clinical diagnostic requirements but where no allergen-specific IgE or skin test reactivity can be demonstrated (Halbert and others 1995; Akdis and others 1999a).

Allergen specific T-cells, furthermore, are not found in these individuals, despite the fact that CLA^+ T-cells dominate the cutaneous inflammatory infiltrate (Akdis and others 1999a; Schmid-Grendelmeier and others 2001).

These findings have lead to the suggestion that there are two forms of atopic dermatitis in humans - an allergic (extrinsic) form with allergen specific IgE and mast cell reactivity; and a non-allergic (intrinsic) form that is not associated with IgE or mast cell reactivity (Halbert and others 1995; Hanifin 1996; Akdis and others 1999a; Tanaka and others 1999). In humans, the clinical and epidemiological features of extrinsic and intrinsic atopic dermatitis are very similar, although the onset of symptoms is later in the intrinsic form (Schmid-Grendelmeier and others 2001).

The pathogenesis of intrinsic atopic dermatitis is unclear. Abnormal lipid metabolism and bacterial ceramidase activity, which decrease epidermal ceramides (Fartasch and Diepgen 1992; Ohnishi and others 1999; Hara and others 2000), reduced δ6-desaturase activity, arachidonic acid and di-homo gamma linoleic acid levels (Hanifin 1996) could compromise epidermal barrier function. Abnormalities in δ6 and δ5 destaurase activity, decreased fat absorption and ultastructural abnormalities in the epidermal lipid barrier have also been observed in atopic dogs (Horrobin and others 1990; van den Broek and Simpson 1990; Scott and others 1997; Taugbol and others 1998; Olivry and others 2000). An impaired cutaneous barrier could enhance inflammatory reactions to irritants and microorganisms (Leung 2000). Auto-antigens could also play a role in the pathogenesis of intrinsic atopic dermatitis (Schmid-Grendelmeier and others 2001).

The predilection sites identified in this study predominantly involve ventral and/or sparsely haired skin. This may expose these sites to percutaneous allergen uptake or

to irritants in the putatively allergic and non-allergic forms respectively. Some of these sites are also subject to increased temperature and humidity, and frictional trauma that may make them particularly vulnerable to environmental allergens, irritants and secondary infection.

The results from this study provide evidence for a non-allergic form of atopic dermatitis that is clinically indistinguishable from allergic atopic dermatitis. However, it is still possible that these dogs are sensitised to allergens not included in the intradermal test and serology panels, and further studies are needed to elucidate the pathogenic mechanisms in these animals.

3.4.2 The role of microbial colonisation in canine atopic dermatitis

The present study shows that the development of chronic lesions in canine atopic dermatitis is associated with secondary microbial infection as *Malassezia* dermatitis or staphylococcal pyoderma significantly increased clinical lesion scores. Colonisation with staphylococci significantly worsened all of the clinical signs except seborrhoea, and *Malassezia* all except scaling and hyperpigmentation. Furthermore, apart from erythema, mean lesion scores at body sites without clinical or cytological evidence of secondary infection were less than 1.0. These findings further indicate that, in its pure form, canine atopic dermatitis is a pruritic and erythematous condition.

Papules were rarely observed in the absence of secondary infection. They are not, however, regarded as a primary lesion in atopic dermatitis, although they are commonly observed in bacterial pyoderma and *Malassezia* dermatitis (Scott and others 2001a). Scaling, which has been associated with both atopic dermatitis and bacterial pyoderma (Scott and others 2001a), was significantly worsened by staphylococcal

infection. In contrast, the degree of scaling was not significantly altered by *Malassezia* dermatitis, in which it is a less common feature (Mason and Evans 1991). Similarly, seborrhoea, which is a feature of both atopic dermatitis and *Malassezia* dermatitis, was significantly worsened by the presence of *Malassezia*, but not staphylococcal pyoderma, where it is less common (Scott and others 2001a). Lichenification and hyperpigmentation frequently complicate chronic atopic dermatitis as well as bacterial pyoderma and *Malassezia* dermatitis (Mason and Evans 1991; Scott and others 2001a). However, in this study, lichenification and hyperpigmentation were strongly associated with microbial infection, suggesting that this plays an important role in the pathogenesis of chronic atopic dermatitis.

Exacerbation of human atopic dermatitis by *Staphylococcus aureus* is associated both with specific IgE and superantigen production (Bunikowski and others 1999; Skov and Baadsgaard 2000). Cutaneous application of lipoteichoic acid isolated from *S. aureus*, furthermore, induced T_{H2}-like inflammation in a mouse model (Matsui and Nishikawa 2002). Specific IgE antibodies against *S. intermedius*, the species most commonly isolated from dogs, have been detected, but their role in canine atopic dermatitis is unclear (Morales and others 1994). *S. intermedius* also produces superantigens (Adesiyun and Usman 1983), but superantigen activity in canine atopic dermatitis has not been studied.

Colonisation with *Malassezia furfur* has been associated with specific IgE and PBMC proliferation in human atopic patients, but no evidence of superantigen activity has been found (Johansson and others 1999). There is evidence that *M. pachydermatis* acts as a conventional allergen in canine atopic dermatitis (Morris and oth-

ers 1998; Nuttall and Halliwell 2001). Other studies have identified major and minor IgG and IgE binding proteins in *M. pachydermatis* (Chen and others 2002).

Clinically and cytologically significant *Malassezia* and staphylococcal infections mostly affected parts of the body previously described as predilection sites in dogs (DeBoer and Marsella 2001). *Malassezia* were most commonly seen in the interdigital skin, axillae, groin, ventral body and ears. Staphylococci were mostly found on the ventral abdomen and groin, with occasional generalised infections. These are also all predilection sites for clinical signs in canine atopic dermatitis (Scott and others 2001a). It appears likely, therefore, that alterations to the microenvironment of atopic skin allows these organisms to establish remote from their mucosal carriage sites (Bond and others 1994; Harvey and Lloyd 1994). Licking enhances seeding of organisms, and excoriation, increased temperature and moisture, altered immune response, or changes in the epidermal lipid profile may all favour colonisation. Once established, these organisms appear to be important in the pathogenesis of lesional canine atopic dermatitis, but further investigation will help to define their role in canine atopic dermatitis.

3.4.3 Reactions to intradermal allergens

At least one positive reaction to each of the 56 allergens was observed in the intradermal test positive atopic dogs, emphasising the wide range of potential environmental allergens. Despite this, reactions to house dust mites, particularly *Dermatophagoides* species, appeared to be the most prevalent. This finding is consistent with data from a 1998 survey at the University of Edinburgh, where 49 and 35 of 57 dogs tested reacted to *D. farinae* and *D. pteronyssinus* respectively (T.J.Nuttall, un-

published observations). However, an earlier study reported that only 62% and 58% of intradermal test positive atopic dogs reacted to house dust mite allergens in Edinburgh and London respectively (Sture and others 1995). The reason for the discrepancy between this and the present study is unknown. It is unlikely that changes in indoor environments and genetic factors could have a made a significant difference in the short time between these studies. Both studies used the same allergen extracts at the same concentrations, although it is possible that the allergen content could have differed. Intradermal test allergens are crude protein extracts that can show significant batch variation in allergenicity and irritancy (Hill and DeBoer 2001). It is possible that frequent sensitisation to human dander reported in the Edinburgh/London survey (Sture and others 1995), which was not observed in this study, could also have influenced the results. A ten-fold greater dilution of human dander is now used, as it was felt the higher concentration could be irritant (R.E.W. Halliwell, K.L. Thoday, P.B. Hill, T.J. Nuttall and S.T. Auxilia, unpublished observations). The prevalence of intradermal test reactions to dust and dust mites in this study was also higher than the 60-70% of positive reactions reported for these allergens in North America (DeBoer 1989; Hill and DeBoer 2001; Scott and others 2001a). These data, however, must be interpreted in light of the high frequency of responses to moulds, pollens and fleas seen in North America (Hill and DeBoer 2001).

Reactions to other mites were less common. *Eurogylphus maynei* is widespread, but much less numerous than *Dermatophagoides* mites (Le Mao and others 1998; Smith and others 1999). This and the forage mites *Acarus, Lepidoglyphus* and *Tyrophagus*, are less important in human (Thomas and others 1998a) and canine atopic diseases (Sture and others 1995).

Responses to other allergens were of much less significance. Reactions to insect allergens were largely accounted for by fleas and *Simulium* species Blackflies. A study in the US similarly found that reactions to insects other than fleas were uncommon (DeBoer 1989). A recent review also concluded that reactions to arthropods other than *Dermatophagoides* mites and fleas are uncommon and that their relevance to atopic dermatitis remains unproven (Sousa and Halliwell 2001). This review also concluded that atopic dermatitis might predispose to flea allergy dermatitis. The low level of sensitisation to fleas among the atopic dogs in this study does not support this conclusion. However, until the true prevalence of flea exposure and sensitisation among atopic and non-atopic dogs in the UK is studied, these findings must be interpreted with care. Reactions to house dust mite and other allergens were also seen in all of the flea allergen positive dogs. None of them, furthermore, had a response to flea control programmes and all had clinical signs that were compatible with atopic dermatitis rather than flea allergic dermatitis.

Reactions to pollen allergens were particularly uncommon. Positive reactions to weed pollens appeared to be more common than reactions to grass or tree pollens, but may be increased by the number of responses to Red Clover (*Trifolium pratense*). This too may be an irritant at the concentration used or could represent a clinically irrelevant reaction. If reactions to this allergen are deleted, the overall response to weed pollens falls to 11.1% and 14.7% of all atopic and intradermal test positive atopic dogs respectively, which are closer to the figures for other pollens.

The frequency of reactions to danders, insect, pollen and mould allergens are consistent with the 1998 survey, suggesting that these figures represent a stable picture of sensitisation in atopic dogs from Scotland and the North of England. Despite this, an

earlier study of intradermal test reactions in Edinburgh and London (Sture and others 1995) found that reactions to tree, grass and weed pollens were seen in approximately 20% of intradermal test positive dogs. Again, the reasons for these discrepancies are not clear, although conifers, the dominant tree species in Scotland, may be less allergenic than deciduous species (Hyde 1956).

The prevalence of pollen reactions in the UK are consistently lower than those reported in North American surveys (Hill and DeBoer 2001; Scott and others 2001a). The reason for this is unclear. There has been little effort to standardise allergen extracts and techniques in veterinary dermatology, although similar allergens are often used at similar concentrations on both sides of the Atlantic. Furthermore, there was a good correlation between reactions to Greer and ARTU tree pollen extracts. However, the low frequency of reactions to tree pollens means that this data should be confirmed with a larger sample size. The relative frequency of pollen reactions could also be influenced by the presence of a few highly allergenic species. The highly allergenic Ragweeds (*Ambrosia* species) are common allergens in the US (Scott and others 2001a). Similarly, pollen reactions in Japanese atopic dogs are dominated by Japanese Cedar (*Cryptomeria japonica*) and Japanese Cypress (*Chamaecyparis obtusa*) (Masuda and others 2000). Short Ragweed (*Ambrosia artemisifolia*) has become locally established in the UK (Mabey 1997), but there are no reports of widespread sensitisation in atopic dogs.

Reactions to intradermally injected allergens do not mimic clinical and histopathological findings in canine atopic dermatitis. Nevertheless, it is accepted that positive reactions demonstrate IgE mediated hypersensitivity to environmental allergens (Hill and others 2001). Despite this, eleven out of the 45 dogs tested in this study did

not react to any allergens. This is higher than the 12% non-response rate reported in a large study of intradermal tests in North America, although only 75% of dogs tested had reactions to allergens other than fleas (DeBoer 1989). Similar results were reported in a study of intradermal tests in Edinburgh and London (Sture and others 1995). In humans, the intrinsic (non-IgE mediated) form accounts for 15-45% of cases of atopic dermatitis (Wollenberg and others 2000; Schmid-Grendelmeier and others 2001).

It was not possible to evaluate six and 24 hour late phase responses in this study. These responses closely resemble atopic dermatitis and may be more clinically relevant (Olivry and others 2001), although less sensitive than immediate responses (Hillier and others 2000). A recent review speculates that this may be due to the effects of dose and route of allergen exposure and/or differential mediator release between immediate responses, late phase responses and spontaneous atopic dermatitis (Hill and others 2001). The relationship between these clearly needs further study.

3.4.4 The epidemiology of canine atopic dermatitis

This study confirms the view that a young age of onset is a feature of canine atopic dermatitis. It is generally accepted that the age of onset lies between six months and three years of age in 75% or more cases of canine atopic dermatitis (Griffin and DeBoer 2001; Scott and others 2001a). The mean age seen in this study was at the upper end of this range, but the figures refer to age at presentation, rather than age of onset. A previous study found that the mean interval from onset of clinical signs to presentation at the University of Edinburgh Hospital for Small Animals was 27 months (Nuttall and others 1998).

The mean age of the healthy dogs was higher than that of the two groups of atopic dogs. There were, however, no significant differences between the three groups and the age range was similar in each case. Age related changes in immune function, including PBMC proliferation and antibody titres, but not delayed type hypersensitivity or *in vitro* cytokine production, are reported in dogs (Kearns and others 1999). Another study reported a decrease in lymphocyte numbers and proliferative responses with age (Greeley and others 1996). However, it is likely that these changes would contribute to intra- rather than inter-group variation in this project.

Males outnumbered females in the intradermal test positive atopic dogs compared to the other two groups, in which the sexes were evenly balanced. However, there were no significant differences in the numbers of male and female dogs in each group. Conflicting reports have suggested both a female and a male bias in canine atopic dermatitis, but these findings have not been consistently observed (Griffin and DeBoer 2001; Scott and others 2001a).

Canine atopic dermatitis is widely regarded as a familial disease. One standard text quotes Labradors, Golden Retrievers, West Highland White Terriers, Boxers, English Setters and German Shepherd Dogs as predisposed breeds (Scott and others 2001a). However, these data were taken from a large number of studies in different countries, where the gene pools may differ. In addition, there is often no reference to the frequency of breeds in the local canine population. A recent review of data controlled for the local population concluded that Beauceron, Boston Terriers, Boxers, Cairn Terriers, Shar Pei, Dalmatians, English Bulldogs, English Setters, Irish Setters, Fox Terriers, Labradors, Labrit, Lhasa Apso, Miniature Schnauzers, Pugs, Scottish Terriers, Sealyham Terriers, West Highland White Terriers, and Yorkshire Terriers were

all predisposed to atopic dermatitis (Griffin and DeBoer 2001). The most common breeds seen in the present study were Labradors and German Shepherd Dogs. This may, however, simply reflect the general canine population as these are two of the most popular dog breeds in the UK (Pet Food Manufacturers Association data; URL - http://www.pfma.com/petownership.htm). In all, the 45 atopic dogs in this study were from 22 breeds, the majority of which were represented by one or two individuals. This suggests that atopic traits are common to a large number of breeds. Alternatively, atopic traits *per se* could be less important than environmental factors. Multicentre studies involving larger numbers of dogs from different breeds and intervention studies looking at the effect that environmental manipulation has on the development of atopic dermatitis would be needed to answer this question. Similar studies are currently underway in human medicine (Custovic and others 1998).

3.4.5 Histopathological findings in atopic and healthy skin

Histopathology of healthy skin in this study was consistent with previous descriptions of normal canine skin (Scott and others 2001b). This suggests that the healthy dogs were not suffering from sub-clinical inflammatory skin disease and were, therefore, appropriate controls.

Non-lesional atopic skin was taken from the flank. This is not a predilection site in canine atopic dermatitis, and is not commonly involved except in severe, generalised disease. Despite this, there was histopathological evidence of mild inflammatory changes. There was also an increase in the number of dermal cells compared to healthy skin, although this did not reach significance. Mild inflammatory changes

and mononuclear cell infiltration have been noted in non-lesional skin from human atopic dermatitis (Thestrup-Pedersen and others 1997; Leung 1999; Leung 2000).

Lesional skin was marked by obvious histopathological changes and a significant increase in the number of dermal cells. This supports the clinical assessment of non-lesional *versus* lesional skin and confirms there is a valid difference between these sites. As these were dogs recruited in a clinical setting, reliable differentiation between acute and chronic lesions proved impossible. However, uncomplicated atopic lesions were selected as far as possible by avoiding obviously excoriated or infected skin. The findings are consistent with previous reports of acute and chronic lesional skin in human (Leung 1999; Leung 2000) and canine (Olivry and others 1997; Sinke and others 1997; Olivry and others 1999a; Olivry and Hill 2001) atopic dermatitis.

Eosinophils were rarely observed, even in lesional skin, although special stains such as sirrius red or immunohistochemistry may have helped to distinguish them. Some atopic dogs exhibit eosinophilic exocytosis and microabcesses, but eosinophils are not as marked a feature of canine compared to human atopic dermatitis (Olivry and others 1997). However, it may be difficult to detect degranulated cells and as their granule contents are highly damaging to surrounding tissues, a very few eosinophils could have a disproportionate effect (Olivry and Hill 2001). A recent study reported marked eosinophil infiltration in the early stages of intradermal allergen induced late phase inflammatory responses (Olivry and others 2001). Further study of the role eosinophil derived mediators play in atopic skin is clearly needed.

In keeping with a previous study (Olivry and others 1997) neutrophils were uncommon. Neutrophil rich infiltrates are thought to be a response to secondary bacterial infection and trauma (Olivry and Hill 2001), which were avoided in this study.

No differences between the histopathology of skin biopsies from intradermal test positive and intradermal test negative animals were seen in this study. Histopathological findings are also similar in extrinsic and intrinsic human atopic dermatitis (Akdis and others 1999a; Schmid-Grendelmeier and others 2001). Fas-Fas ligand mediated apoptosis of keratinocytes induced by infiltrating T-cells has recently been associated with the development of lesional atopic dermatitis (Trautmann and others 2000). The mononuclear cell infiltrate into lesional atopic skin observed in this study may therefore play a crucial role in pathogenesis. This may disrupt the epidermal barrier and induce lesions common to both allergic and non-allergic atopic dermatitis. The difference between the two would then lie with the factors that recruit mononuclear cells to the skin.

3.4.6 Conclusion

This study has demonstrated that canine atopic dermatitis is largely a pruritic and erythematous disease that predominantly affects ventral, sparsely haired skin. The development of chronic lesions is strongly associated with secondary bacterial and *Malassezia* infections. This study also provides evidence for the existence of clinically indistinguishable allergic and non-allergic forms of canine atopic dermatitis.

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

4.1 Introduction

It is clear from the findings in Chapter 3 and other studies (reviewed in Hill and De-Boer 2001) that the most important allergens in canine atopic dermatitis are the house dust mites *D. pteronyssinus* and *D. farinae*. More than 16 *Dermatophagoides* allergens relevant to humans have been identified (Chapter 1; Table 1.1), but the most important are group 1 and group 2 allergens (Thomas and Smith 1999). The spectrum of allergens relevant to dogs is less clear. Several studies suggest that high molecular weight proteins are more important allergens than the low molecular weight group 1 and 2 proteins (Noli and others 1996; McCall and others 2000; Shaw 2000), whereas other studies report a high frequency of reactions to group 1 and 2 allergens (Masuda and others 1999; McCall and others 1999). The antisera used in these studies could, however, have influenced the results as different antisera can vary in specificity and sensitivity (Steward and Male 1998). In addition, SDS-PAGE was originally described using reducing conditions (Laemmli 1970), which can denature IgE epitopes and reduce IgE binding to group 1 and 2 *Dermatophagoides* allergens (Lombardero and others 1990).

The allergen extracts used may also be important. The allergen content varies depending on the source, mite life cycle and proportion of mites to media (O'Hehir 1988; Le Mao and others 1998). Furthermore, IgE and T-cell epitope polymorphisms

have been found in a range of *Dermatophagoides* allergens (Smith and others 1997; Le Mao and others 1998). This has important implications for research, diagnosis and immunotherapy, as different mite extracts may not contain all relevant epitopes. It is thus difficult to compare results between studies using different reagents and mite extracts. The aim of this study, therefore, was to characterise and compare IgE binding proteins detected on both reduced and non-reduced western blots of *Dermatophagoides* extracts from a number of sources using both monoclonal and polyclonal anti-canine IgE antibodies.

4.2 Materials and Methods

4.2.1 Study population and sample collection

Dogs with atopic dermatitis (n=33) and healthy dogs (n=26) were recruited and clinical data recorded according to the inclusion criteria described in Chapter 2.1. Serum samples were collected and stored as described in Chapter 2.2.1.

4.2.2 SDS-PAGE analysis of the *Dermatophagoides* extracts

The sources of the crude *D. farinae, D. pteronyssinus* and *D. microceras* extracts, and the purified group 1 and group 2 allergens are listed in Chapter 2, Table 2.2. The freeze-dried extracts were reconstituted to 1mg/ml in sterile PBS as described in Chapter 2.3. SDS-PAGE was performed using 10µg of each extract in reducing and non-reducing loading buffer as outlined in Chapter 2.4. Duplicate gels were stained with 2.5% Coomassie Blue and Silver and imaged as described in Chapter 2.4.3.

4.2.3 Immunoblotting

western blots of each extract diluted in reducing and non-reducing buffer were prepared and probed with monoclonal or polyclonal anti-canine IgE (Chapter 2.5).

4.2.3.1 Validation

A single allergen source (Greer *D. farinae* under reducing conditions) and a known positive serum sample were initially used to validate the technique. The sample was strongly reactive to *D. farinae* in ELISAs and on western blots (T.J. Nuttall, T.-M. Lian and P. Cameron; unpublished data). The appropriate channels of the MN28 miniblotter® (Chapter 2.5.2.2) were incubated for one hour at room temperature with

85µl of a 1/5, 1/10, 1/20 or 1/50 dilution of serum. Each serum dilution channel was then incubated for one hour at room temperature with a 1/50, 1/100, 1/500, 1/1000 or 1/5000 dilution of either the mouse monoclonal or goat polyclonal anti-canine IgE. The appropriate portions of each membrane were incubated at room temperature for one hour with 20ml of the horseradish peroxidase conjugated bovine monoclonal anti-mouse IgG or mouse monoclonal anti-goat IgG diluted 1/500, 1/1000, 1/5000 or 1/10,000 respectively. The membranes were then developed and imaged as described (Chapter 2.5.2.3). Blanks and control channels with no serum, no anti-canine IgE or no anti-IgG were included in each assay. Duplicate blots were performed using the positive control serum heated at 56°C for one hour. The absence of binding to these blots confirmed the specificity of the both the polyclonal and monoclonal antibodies for heat labile IgE epitopes (Tizard 1996).

Optimum binding was seen with serum diluted 1/5, the mouse monoclonal anti-canine IgE diluted 1/500, the goat polyclonal anti-canine IgE diluted 1/100, the bovine anti-mouse IgG diluted 1/1000 and the mouse anti-goat IgG diluted 1/5000. There was no non-specific binding to serum or allergens at these dilutions, which were used in the rest of the study.

4.2.3.2 Screening of sera from atopic and healthy dogs for allergen specific IgE

Western blots of each allergen were prepared and blocked as previously described. The appropriate channels of the MN28 miniblotter® were incubated for one hour at room temperature with 85µl of a 1/5 dilution of serum, then 85µl of either a 1/500 dilution of the mouse monoclonal or a 1/100 dilution of the goat polyclonal anti-

canine IgE. The membranes were then incubated at room temperature for one hour with 20ml of the horseradish peroxidase conjugated bovine monoclonal anti-mouse IgG or the mouse monoclonal anti-goat IgG diluted 1/1000 and 1/5000 respectively. The membranes were then washed, developed and imaged as described previously. Blanks and control channels with no serum, no anti-canine IgE and positive control serum were included in each assay.

4.2.4 Data Analysis

Four replicates of each allergen extract immunoblot were generated for both atopic and healthy sera. These were reducing buffer with monoclonal anti-canine IgE; reducing buffer with polyclonal anti-canine IgE; non-reducing buffer with monoclonal anti-canine IgE; and non-reducing buffer with polyclonal anti-canine IgE. The numbers of positive sera identified on each of these immunoblots were compared using Fisher's exact test with significance set at $p<0.05$ (Instat®). Mann-Whitney tests were used where appropriate to compare the number of positive atopic and healthy sera that recognised each allergen extract (Instat®).

Standard curves for each blot were constructed by plotting the log10 molecular weight of each molecular weight marker against the distance migrated by each marker divided by the distance migrated by the loading dye. Regression equations from the standard curves (MS Excel 2000®) were used to calculate the molecular weight of the allergens recognised by each serum sample. The mean molecular weight of each allergen was plotted against the percentage of positive sera that recognised the allergen on the reduced and non-reduced blots of each extract. Major allergens were defined as those recognised by 50% or more of positive sera.

4.3 Results

4.3.1 Intradermal tests

The majority of the dogs with atopic dermatitis had positive reactions to *D. farinae* and *D. pteronyssinus* (Table 4.1). Occasional reactions were also seen to a variety of other mite, insect, pollen and mould extracts. Only four dogs out of the 33 with clinical atopic dermatitis in this study did not react to a least one allergen. No dog reacted to *D. pteronyssinus* in the absence of a reaction to *D. farinae*. Two dogs, however, had reactions to *D. farinae*, but not *D. pteronyssinus*.

Table 4.1 – Intradermal test (IDT) reactions to *Dermatophagoides farinae* and *D. pteronyssinus* in dogs with atopic dermatitis (n=33)

Allergen extract	IDT result	
	Positive	Negative
D. farinae	24	9
D. pteronyssinus	22	11

4.3.2 SDS-PAGE of the *Dermatophagoides* extracts

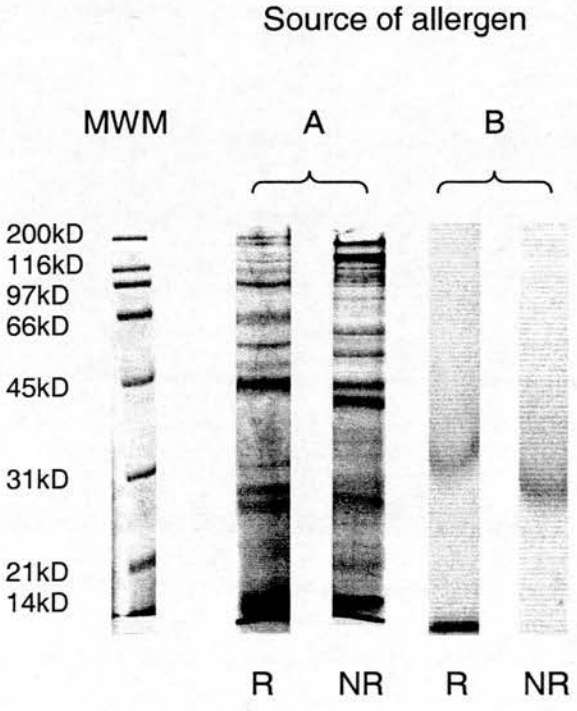
The protein content varied widely between all the extracts, even those from the same source. Protein bands were clearly identified on gels of all the extracts (Figure 4.1a-e). Multiple bands were identified on the Greer *D. farinae*, and Greer and AP *D. pteronyssinus* gels, although the migration patterns were not identical. Far fewer bands, which were predominantly low molecular weight proteins, were seen on the ALK *D. farinae*, ALK and IG *D. pteronyssinus*, and ALK *D. microceras* gels.

Prominent bands of the expected molecular weight were seen with the purified AP and IG Der p 1, and AP Der p 2 extracts. Clear bands of the appropriate size were

seen with the purified ALK Der f 1 and Der f 2 extracts, although faint lower and higher molecular weight bands were also present, especially in the Der f 2 extract. Different migration patterns were seen on non-reduced gels, although the overall pattern was similar to the corresponding reduced gel. There were few differences between duplicate gels stained with silver and Coomassie blue.

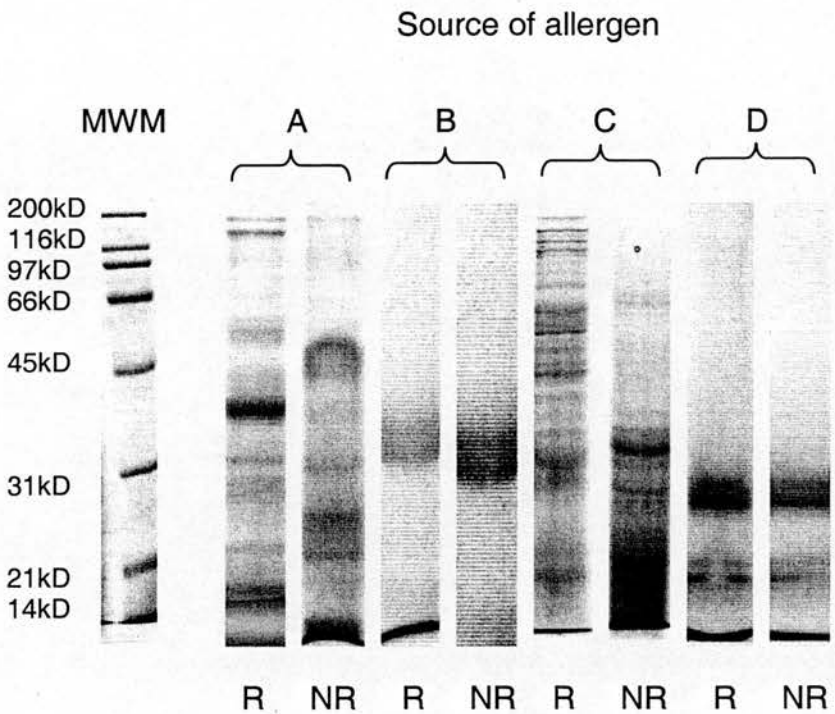
Figure 4.1 – SDS-PAGE analysis of crude and purified *Dermatophagoides* extracts (2.5% Coomassie blue stain transilluminated by white light)

a – *Dermatophagoides farinae*

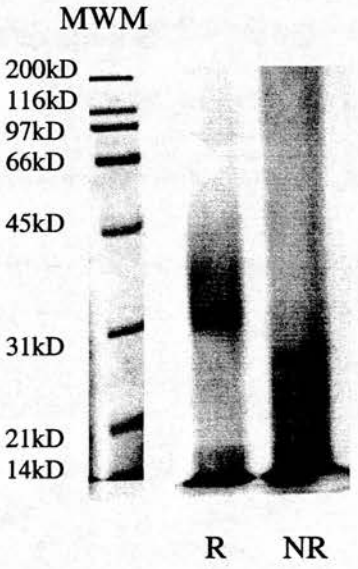


MWM - Molecular weight markers (Broad range; Biorad Laboratories); R - Reducing buffer; NR - Non-reducing buffer. Source of allergens: A – Greer Laboratories; B – ALK-Abéllo.

b – *Dermatophagoides pteronyssinus*

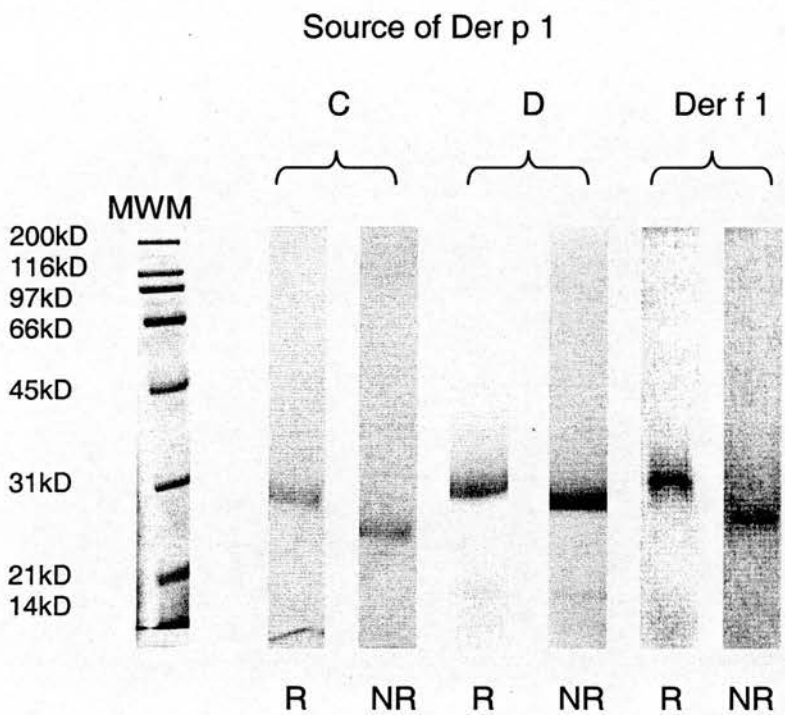


MWM - Molecular weight markers (Broad range; Biorad Laboratories,); NR - Non-reducing buffer. Source of allergens: A – Greer Laboratories; B – ALK-Abéllo; C – Allergo-Pharma; D – Immunobiology Group.



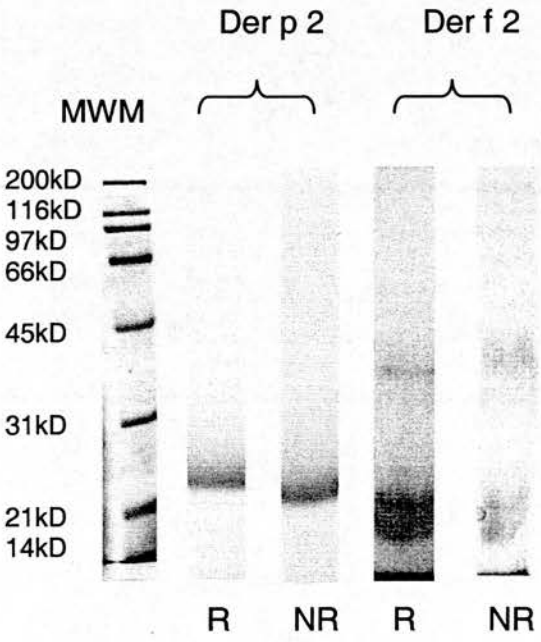
MWM - Molecular weight markers (Broad range; Biorad Laboratories); NR - Non-reducing buffer. Source of allergen: ALK-Abéllo.

d – Purified group 1 allergens



MWM - Molecular weight markers (Broad range; Biorad Laboratories); R - Reducing buffer; NR - Non-reducing buffer. Source of allergens: C – Allergo-Pharma; D – Immunobiology Group; Der f 1 - ALK-Abéllo.

e – Purified group 2 allergens



MWM - Molecular weight markers (Broad range; Biorad Laboratories); R - Reducing buffer; NR - Non-reducing buffer. Source of allergens: ALK-Abéllo.

4.3.3 Immunoblotting

Only one serum sample from an atopic dog that had no response to house dust mites on skin testing detected *D. farinae* allergens under all blotting conditions (i.e. reducing and non-reducing buffer, mono- and polyclonal anti-canine IgE). Two further sera from IDT negative dogs detected allergens on single blots using reducing buffer/monoclonal anti-canine IgE and non-reducing buffer/polyclonal anti-canine IgE respectively. One sample detected *D. pteronyssinus* allergens using reducing buffer and monoclonal anti-canine IgE only. Representative examples of the immunoblots are illustrated in Figure 4.2.

There were no significant differences in the number of positive sera identified using either polyclonal or monoclonal anti-canine IgE for any of the extracts under reducing or non-reducing conditions (Table 4.2). The results were therefore combined to give four replicates (i.e. monoclonal anti-canine IgE/reduced blot, monoclonal anti-canine IgE/non-reduced blot, polyclonal anti-canine IgE/reduced blot and polyclonal anti-canine IgE/non-reduced blot) for each allergen extract in the following analyses.

Figure 4.2 - Representative immunoblots

a - Greer *D. farinae*, atopic sera, polyclonal anti-IgE, non-reducing buffer; b - Greer *D. farinae*; healthy sera, polyclonal anti-IgE, non-reducing buffer; Greer *D. farinae*, atopic sera, monoclonal anti-IgE, reducing buffer; Greer *D. farinae*, healthy sera, monoclonal anti-IgE, reducing buffer; e - IG Der p 1, atopic sera, polyclonal anti-IgE, non-reducing buffer; f - AP Der p 2, atopic sera, polyclonal anti-IgE, non-reducing buffer;

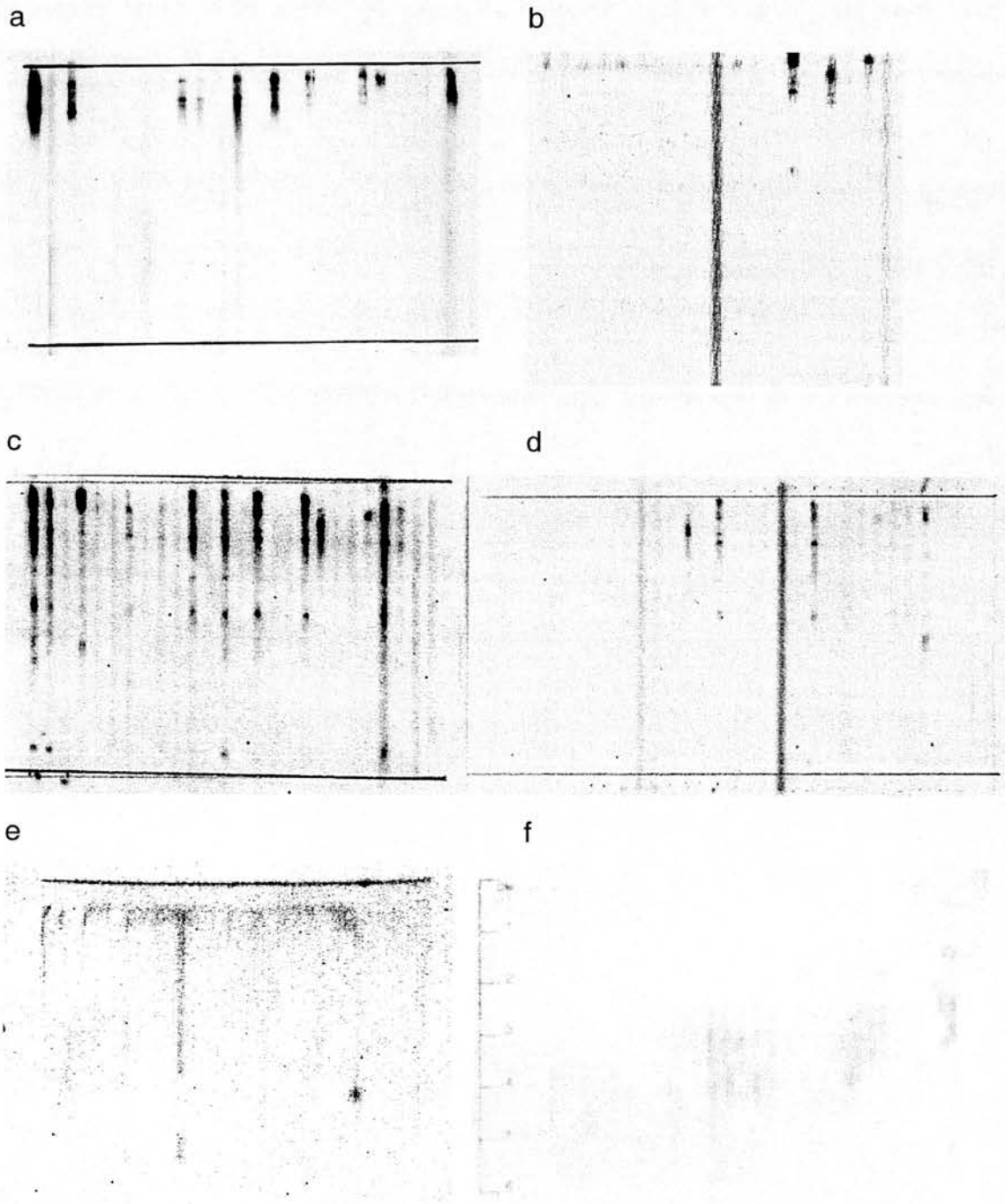
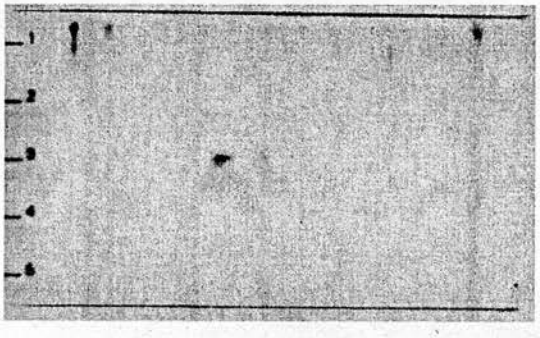


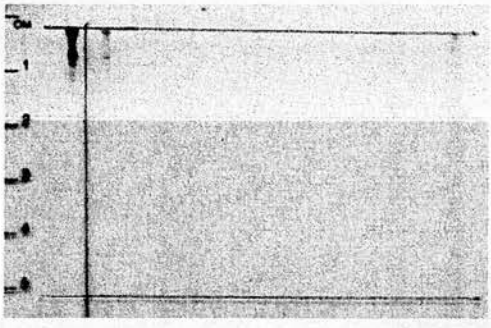
Figure 4.2 contd. - Representative immunoblots

g - ALK Der f 1, atopic sera, polyclonal anti-IgE, non-reducing buffer; h - g - ALK Der f 2, atopic sera, polyclonal anti-IgE, non-reducing buffer; i - Greer *D. pteronyssinus*, atopic sera, monoclonal anti-IgE, reducing buffer; j - Greer *D. pteronyssinus*, healthy sera, monoclonal anti-IgE, reducing buffer.

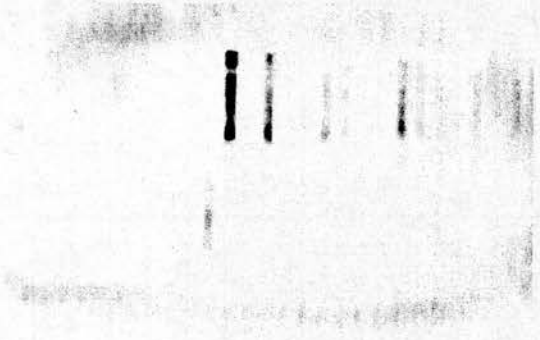
g



h



i



j

Table 4.2 – Percentage of atopic (n=33) and healthy (n=26) sera that bound to western blots of crude and purified *Dermatophagoides* allergens

DF - *Dermatophagoides farinae*; DP - *D. pteronyssinus*; DM - *D. microceras*; Greer - Greer Laboratories; ALK - ALK-Abéllo; AP - Allergo-Pharma; IG – Immunobiology Group; Mab – monoclonal anti-canine IgE; Pab – polyclonal anti-canine IgE.

	Atopic sera				**Healthy sera**			
	Reduced blot		**Non-reduced blot**		**Reduced blot**		**Non-reduced blot**	
Allergen extract	**Mab**	**Pab**	**Mab**	**Pab**	**Mab**	**Pab**	**Mab**	**Pab**
Greer DF	67	58	52	58	23	12	15	11
ALK DF	50	32	25	42	15	15	4	8
Greer DP	50	35	50	24	8	32	4	4
ALK DP	30	26	28	32	19	4	15	22
AP DP	48	48	32	36	4	7	16	16
IG DP	7	0	0	0	4	0	0	0
ALK DM	21	8	4	4	4	7	4	7
AP Der p 1	0	0	0	0	0	0	0	0
IG Der p 1	0	0	0	4	0	0	4	0
AP Der p 2	0	0	0	0	0	0	0	0
ALK Der f 1	12	8	29	12	0	0	0	0
ALK Der f 2	12	20	15	11	0	0	0	0

4.3.3.1 *IgE binding to* D. farinae *extracts*

Significantly more atopic than healthy sera recognised IgE binding proteins on the Greer and ALK *D. farinae* blots ($p<0.05$) (Figure 4.3a). In addition, significantly more atopic sera bound to the Greer than to the ALK extract ($p<0.05$).

4.3.3.2 *IgE binding to the* D. pteronyssinus *extracts*

Significantly more atopic than healthy sera recognised IgE binding proteins in each of the Greer, ALK and AP *D. pteronyssinus* extracts ($p<0.05$) (Figure 4.3b). There was no significant difference in the frequency of binding between these extracts. However, significantly fewer atopic sera recognised the IG compared to the other extracts ($p<0.05$), nor was there any significant difference between the numbers of atopic and healthy sera that bound to this extract. Significantly more atopic sera recognised the Greer *D. farinae* extract compared to the Greer *D. pteronyssinus* extract ($p<0.05$), but there was no significant difference between the ALK *D. farinae* and *D. pteronyssinus* extracts. No serum sample from a *Dermatophagoides* skin test positive dog bound to *D. pteronyssinus* in the absence of binding to *D. farinae*.

4.3.3.3 *IgE binding to the* D. microceras *extract*

There was no significant difference in the number of atopic and healthy sera that bound to the *D. microceras* blots (Figure 4.3c). Significantly fewer atopic sera, furthermore bound to the ALK *D. microceras* extract than to the ALK *D. farinae* and *D. pteronyssinus* extracts ($p<0.05$).

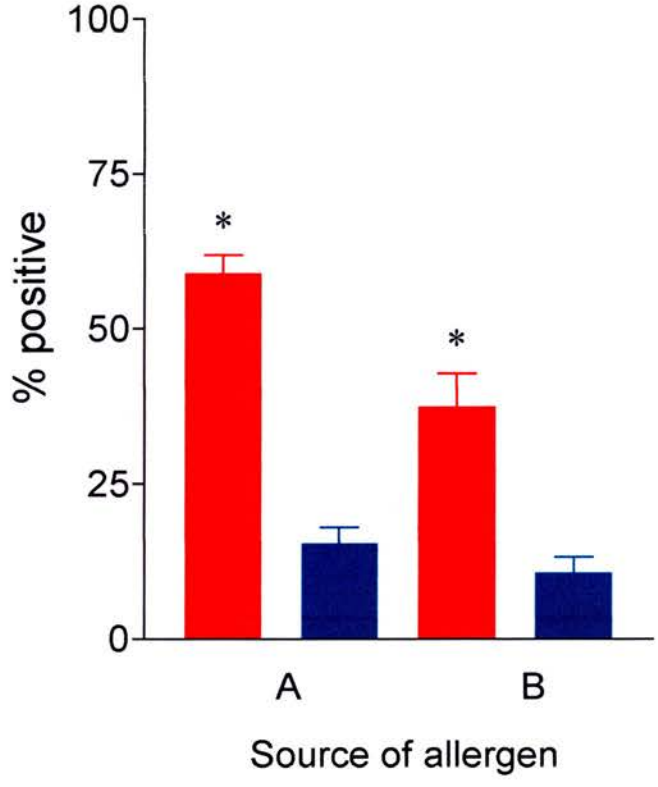
4.3.3.4 *IgE binding to the purified Group 1 and 2 allergens*

A few atopic sera bound to western blots of the ALK Der f 1 and Der f 2 extracts (range 8-29%) (Table 4.2). However, this was largely accounted for by binding to high molecular weight allergens that were also present in these extracts rather than to low molecular weight Der f 1 and Der f 2. Very few sera recognised any of the other purified group 1 and 2 extracts (range 0-4%) (Table 4.2) and there was no significant difference in binding between atopic and healthy sera. In addition, significantly fewer atopic sera bound to any of the group 1 or 2 allergen preparations than to the appropriate crude *D. farinae* or *D. pteronyssinus* extracts ($p<0.05$).

Figure 4.3 - Percentage of atopic and healthy sera that bound to western blots of *Dermatophagoides* allergens

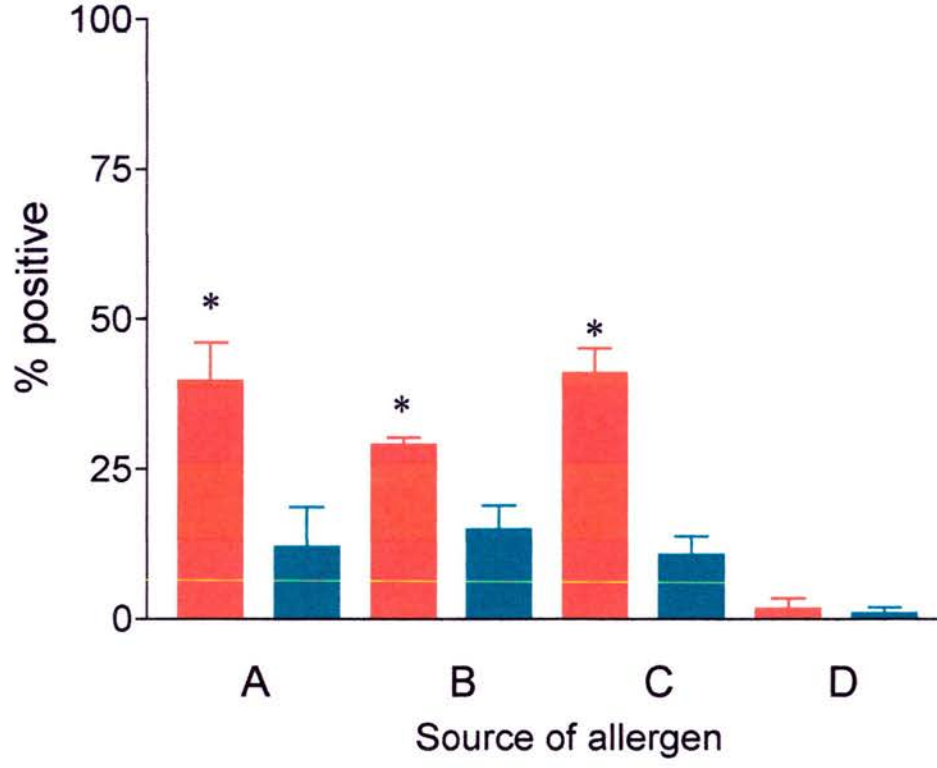
a – *Dermatophagoides farinae*

Atopic sera - ■ ; Healthy sera - ■; A – Greer Laboratories; B - ALK-Abéllo; Bar – sem; * - $p<0.05$.



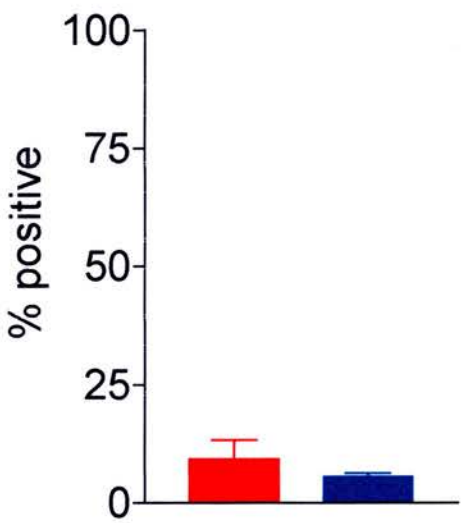
b – *Dermatophagoides pteronyssinus*

Atopic sera - ■ ; Healthy sera - ■; A – Greer Laboratories; B - ALK-Abéllo; C - Allergo-Pharma; D – Immunobiology Group; Bar – sem; * - $p<0.05$.



c – *Dermatophagoides microceras*

Bar - sem; ■ - Atopic sera; ■ - Healthy sera.



4.3.4 Major and minor allergens

A range of major allergens from 44kD to 150kD were identified on blots of the Greer *D. farinae* and *D. pteronyssinus* extracts under reducing conditions. No major allergens under 98kD were identified on blots under non-reducing conditions. Major allergens at 97-98kD and 103-104kD were common to both reduced and non-reduced blots of *D. farinae* and *D. pteronyssinus*. A number of minor allergens from 15-150kD were identified in both *D. farinae* and *D. pteronyssinus*. The distribution of minor allergens was not markedly different between the reduced or non-reduced blots (Table 4.3; Figure 4.4 a and b). Results for the other *D. farinae*, *D. pteronyssinus*, and *D. microceras* extracts were very similar.

Table 4.3 – Major and minor allergens identified in the Greer *Dermatophagoides farinae* and *D. pteronyssinus* extracts

a. *D. farinae*

Major allergens (kD)		Minor allergens (KD)	
Reduced blot	Non-reduced blot	Reduced blot	Non-reduced blot
151	139	116	151
134	103	65	120
104	98	32	63
97		25	28
84		14	21
44			

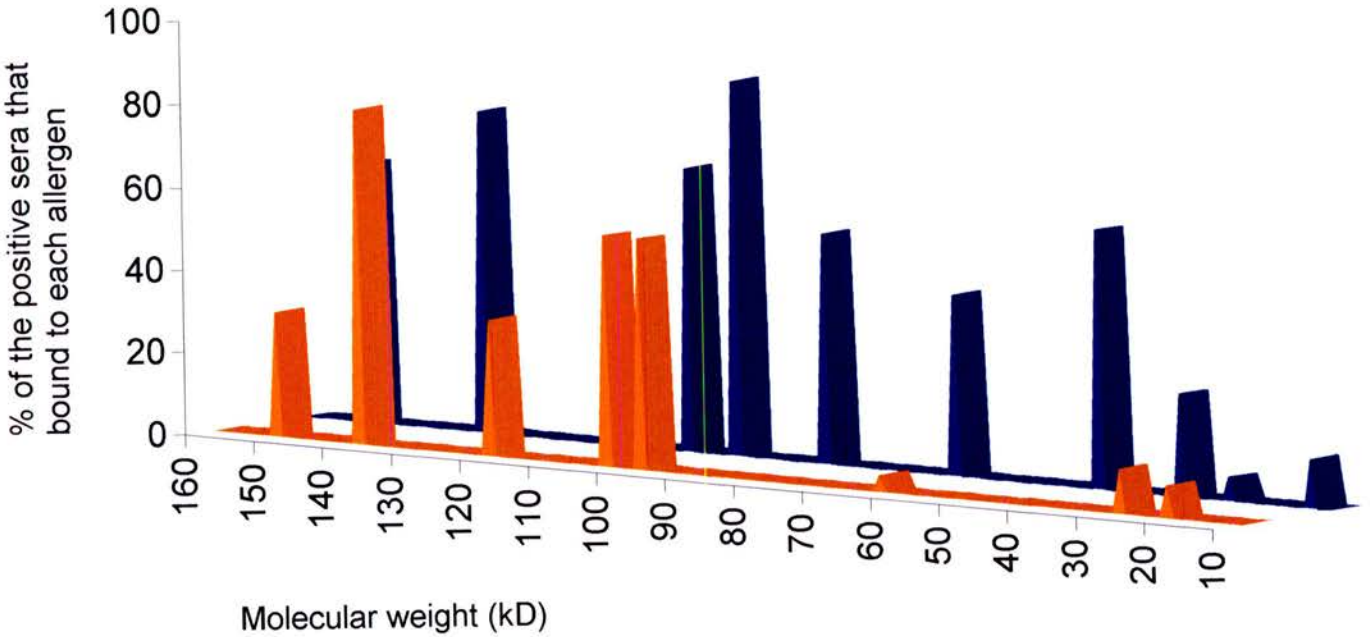
b. *D. pteronyssinus*

Major allergens (kD)		Minor allergens (kD)	
Reduced blot	Non-reduced blot	Reduced blot	Non-reduced blot
103	104	115	150
97	98	85	121
69		34	85
45		25	65
		17	27
			15

Figure 4.4 – Major and minor allergens identified in the Greer *Dermatophagoides* extracts (bars greater than 50% indicate major allergens)

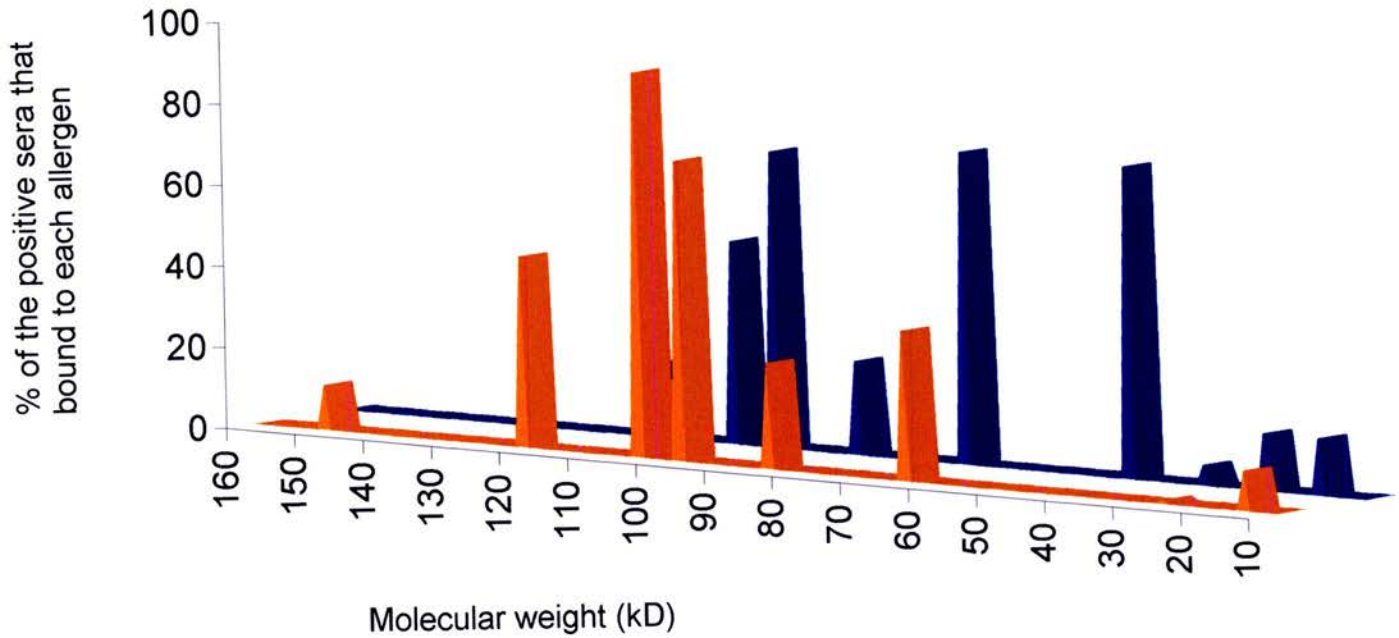
a – *Dermatophagoides farinae*

- Non-reducing buffer; - reducing buffer



b – *Dermatophagoides pteronyssinus*

- Non-reducing buffer; - reducing buffer



4.4 Discussion

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

This study characterised in detail the pattern of recognition of major and minor *Dermatophagoides* allergens by canine atopic sera. Major allergens common to *D. farinae* and *D. pteronyssinus* were detected as double bands at 97-98kD and 103-104kD on both reduced and non-reduced blots. These bands seem relatively resistant to reduction and are probably the double band previously reported as a major allergen in studies using anti-canine IgGd and recombinant FcεRIα (Noli and others 1996; McCall and others 2000). This was recently sequenced and identified as a chitinase named Der f 15 (McCall and others 2000). An unidentified 95-101kD allergen and a 98kD major allergen structurally similar to paramyosin (termed Der f 11) were recently identified on *D. farinae* blots probed with human sera (Le Mao and others 1998; Tsai and others 1999). Further analysis is needed to determine if these are independent allergens, or whether canine and human atopic sera recognise similar high molecular weight allergens.

The canine *D. farinae* major allergen detected at 134-139kD in this study has no obvious homologue. The 98kD chitinase is heavily glycosylated with a native protein molecular weight of approximately 63kD (McCall and others 2000). Multiple bands can be associated with variably glycosylated allergens (Le Mao and others 1998), so this may represent an alternately glycosylated form of the 97-104kD allergen. Other major allergens at 84-85kD, 65-69kD and 44-45kD were only recognised on reducing blots, suggesting these are fragments or denatured forms of the larger allergens. However, it is possible that the 65-69kD allergen is similar to a 60kD allergen re-

cently described in *D. farinae* (Weber 2001) or the native Der f 15. Most allergens possess a number of different IgE epitopes (Bufe 1998), which in this case appear to be intact and located on one or more of these smaller fragments. Degradation products have also been identified on *D. farinae* blots probed with human sera (Le Mao and others 1998). The distribution of minor allergens was similar on both reduced and non-reduced blots. Only a low frequency of binding to low molecular weight bands representing group 1 and 2 allergens was seen. Other minor allergens were identified at 115-121kD and 150-151kD. Further study is required to determine whether these are independent allergens or alternately glycosylated forms of the 97/104kD allergen.

These results also show that, in contrast to humans, group 1 and 2 *Dermatophagoides* allergens are of limited relevance to canine atopic dermatitis. There was little binding of atopic or healthy sera to the IG *D. pteronyssinus* extract, which is prepared from spent mite media and largely consists of Der p 1 and Der p 2 (G. Hoyne, Immunobiology Group, University of Edinburgh Centre for Inflammation Research; personal communication), and very few sera recognised any of the purified group 1 and 2 allergens from *D. farinae* and *D. pteronyssinus*. Reducing conditions can decrease IgE binding to group 1 and 2 allergens (Lombardero and others 1990; Olsson and others 1998), but no difference was seen between reduced and non-reduced blots in this study. No reliable positive control canine sera were available for these allergens, but appropriate protein bands were identified on each blot by staining with Coomassie Blue. Furthermore, these allergens have been used in immunoblotting with human sera and proliferation studies with human and murine PBMCs (G. Nordskov Hansen, ALK-Abello; O. Cromwell, Allergo-Pharma; N. Savage, Immunobiology Group,

University of Edinburgh Centre for Inflammation Research; personal communications). The few atopic sera that bound to the Der f 1 and Der f 2 blots mostly recognised the 97-104kD double band discussed earlier. No contamination at this position was evident on the stained gels and blots, but enough allergen was obviously present for detection by strongly reactive sera. Another study reported that Der f 1 and Der f 2 extracts that elicited positive intradermal tests bound IgGd in ELISAs but not western blots (Noli and others 1996). Impurities are easily identified on western blots, where protein bands are separated by mass, but they could lead to false positive results where proteins are not separated. This may explain the high frequency of binding of canine atopic sera to group 1 and 2 allergens reported in a study using dot blots and ELISAs (Masuda and others 1999).

D. farinae and *D. pteronyssinus* seem to be the major indoor allergens for atopic diseases in both dogs and humans. If certain proteins are held to have intrinsic allergenic properties (Comoy and others 1999), it is at first sight perhaps surprising that the spectrum of relevant allergens should differ between dogs and humans. IgE production is dependent on T-helper cells that recognise short sequences of 10-15 amino acids complexed with MHCII molecules. These T-cell epitopes are derived from processing of allergen by APCs, which profoundly influences the subsequent specificity and quantitative nature of immune responses (Carrasco-Marín and others 1998; Maekawa and others 1998; Huby and others 2000). T-cell epitopes are also MHC class II restricted (Renz 1995; van Neerven and others 1996). The amino and carboxyl termini of these peptides (peptide flanking residues), furthermore, also influence T-cell activation (Carson and others 1999). Alterations in peptide flanking residues can switch subsequent T-cell responses from stimulation to anergy. It is possi-

ble that biological variation in antigen processing between individuals could yield peptides with varying peptide flanking residues and T-cell responses. If canine APCs, therefore, present a different array of peptides or have T-cell receptor and MHCII repertoires that are very distinct from humans, the specificity of allergen recognition could also differ.

Allergenic proteins tend to be ovoid, stable in the environment and possess disulphide bonds that contribute to the conformational stability of IgE epitopes (Smith and others 1997; Huby and others 2000). Many allergens are also glycosylated, which can enhance binding to IgE and increase uptake by APCs (Huby and others 2000). Furthermore, the biological activity of many allergens is thought to contribute to their allergenicity (Huby and others 2000). Der p 1 degrades tight junctions, cleaves α1-antitrypsin, CD23 and CD25 (the IL-2 receptor), and can directly activate mast cells and basophils (Schulz and others 1999; Wan and others 2000). This increases epithelial permeability, enhances protease activity, IgE production, inflammatory mediator release and inhibits T_{H1} differentiation (Bufe 1998; Shakib and Gough 2000). In addition, the 98kD paramyosin Der f 11 can inhibit complement C1, which may influence subsequent immune responses (Tsai and others 1999). The 98kD chitinase Der f 15 appears to be glycosylated, but it is unknown if any of the allergens identified with canine sera are biologically active or what their effects *in vivo* might be. It is difficult to extrapolate from studies in humans and rodents as enzyme activity may be substrate restricted and species specific. Furthermore, even though Der p 1 and Der f 1 are minor allergens in canine atopic dermatitis, it is possible that they could affect inflammatory responses in a non-immunogenic fashion (Schulz and others 1999). In mice, the proteolytic activity of Der p 1 enhances the

IgE response to unrelated antigens (Gough and others 2001). Studies on the *in vivo* biological activity of the 98/104kD allergen, and the effect of group 1 and 2 allergens on canine skin are therefore warranted.

4.4.1 *Dermatophagoides* specific IgE in atopic and healthy canine sera

Significantly more atopic sera than healthy sera bound to allergens in all the whole mite preparations of *D. farinae* and *D. pteronyssinus*, whereas the few sera that recognised *D. microceras* were equally distributed between atopic and healthy dogs. This suggests that IgE responses in atopic dogs are either specific to individual *D. farinae* and *D. pteronyssinus* allergens or to cross-reacting allergens present only in these two species. There is generally 80-90% homology between *D. farinae* and *D. pteronyssinus* allergens (Thomas and Smith 1999). This study did not discriminate between independent sensitisation and cross-reaction, but a cross-inhibition study with canine sera found that *D. farinae* and *D. pteronyssinus* allergens strongly cross-react (Masuda and others 1999). The absence of intradermal test reactivity and IgE titres to *D. pteronyssinus* independent of *D. farinae* in this study also suggests that separate sensitisation to *D. pteronyssinus* is uncommon in canine atopic dermatitis.

These results show that *Dermatophagoides* specific serum IgE antibodies were largely restricted to sensitised atopic dogs. There was good correlation between skin tests and immunoblotting among the atopic dogs, although it was not ethically possible to perform skin tests in the healthy dogs. Using intradermal testing as the standard to detect sensitisation, immunoblots were highly specific, but less sensitive for detecting sensitisation to both *D. pteronyssinus* and *D. farinae*. Reasons for this could include gel separation and blotting making allergens less available and allergen

specific IgG blocking IgE binding. IgG was not measured in this study but generally parallels allergen specific IgE in canine sera (Day and others 1996).

Only a few skin test negative atopic dogs had one or more positive immunoblots, which may have been due to technical problems. They could also be genuine false positives as allergen specific IgE has been reported in healthy dogs and skin test negative atopic dogs (Day and others 1996; Halliwell and others 1998). A number of healthy dogs also had *Dermatophagoides* specific IgE. It remains to be proven whether these dogs develop sub-clinical sensitisation or whether there are isoforms of canine IgE or FcεRI with different binding affinities. One study reported that a high proportion of healthy dogs have positive skin tests (Halliwell and others 1998), suggesting that downstream mechanisms were of more importance in tolerance.

4.4.3 The allergen content of different *Dermatophagoides* extracts

Recognition of the *D. farinae* extracts by atopic sera was influenced by the source of the allergen. The SDS-PAGE migration patterns also revealed differences in the allergen content between each extract. This could be due to variable recovery of allergens between culture and extraction protocols, but there may be significant differences in the expression of certain allergenic proteins between the various *Dermatophagoides* species and strains used for allergen extracts (O'Brien and others 1995; Smith and others 1997; Le Mao and others 1998). Polymorphisms of these allergens could also contribute to the observed variation, but it is unlikely that relatively minor amino acid substitutions or deletions would make much difference to the SDS-PAGE banding pattern. There was, however, no difference in binding to the different *D. pteronyssinus* extracts, despite a similar variation in protein migration patterns.

It is widely accepted that *D. farinae* is a more important allergen to dogs, even though less common than *D. pteronyssinus* in the UK (Sture and others 1995). However, a recent study in a warm and humid area of Brazil thought to be low in *D. farinae* found equal levels of Der f 1 and Der p 1 in house dust. The authors speculated that the ostensible low prevalence of *D. farinae* in some studies may be due to seasonal population fluctuations (Sopelete and others 2000). The apparent importance of *D. farinae* in canine dermatology could also reflect the concentration of key allergens in widely used extracts (Thomas and Smith 1999). However, a recent study has shown that Der f 1 and Der p 1 have different protease activity and substrate preferences, which could influence their relative importance in different species (Pernas and others 2000). Further work to define and standardise the allergen content of *Dermatophagoides* extracts is clearly needed.

4.4.4 Allergen identification on reduced and non-reduced blots using polyclonal and monoclonal anti-canine IgE

The differences in identification of major and minor allergens on reducing and non-reducing blots suggest that results from reducing blots should be interpreted with care. The differences in the pattern of protein migration between reducing and non-reducing gels for each allergen extract show that reducing conditions can profoundly affect the protein structure of *Dermatophagoides* allergens. For example, reducing conditions increased the apparent size of the purified group 1 and 2 allergens by approximately 5-10kD in this and prior studies (Lombardero and others 1990). Reducing agents also denature IgE epitopes (Lombardero and others 1990). Despite this, there were no significant differences in the numbers of atopic and healthy sera that

bound to reducing and non-reducing blots of each allergen, suggesting that both techniques are equally sensitive.

Both polyclonal and monoclonal anti-canine IgE antibodies were used in this study. Monoclonal reagents are generally regarded as more specific, whereas polyclonal reagents can be more sensitive (Steward and Male 1998). Nevertheless, there was no significant difference in sensitivity or specificity between the antisera in detecting *Dermatophagoides* specific IgE in atopic and healthy canine sera.

4.4.5 Conclusion

This study has shown that major *Dermatophagoides* allergens in canine atopic dermatitis are restricted to proteins greater than 95kD. Group 1 and 2 allergens appear to be of limited importance. Using purified and standardised major and minor allergens will be important in understanding the role of mononuclear cells, identifying immunodominant peptides and in the diagnosis and treatment of canine atopic dermatitis.

5. Isolation and culture of canine peripheral blood mononuclear cells

5.1 Introduction

Lymphocyte responses to specific allergens can be investigated by isolating and culturing PBMCS, which are separated from erythrocytes and granulocytes by density centrifugation over 1.077 (human) - 1.083 (rodent) g/L ficoll solutions (Steward and Male 1998). Density centrifugation over 1.077 g/L ficoll has been used to isolate canine PBMCs in a number of studies (Kristensen and others 1982a; Martínez-Moreno and others 1995; Corato and others 1997; Poitout and others 1997). However, other studies report that density centrifugation results in contamination with granulocytes, which in dogs have a similar density to PBMCs (Letwin and Quimby 1987; Rivas and others 1995). These authors used carbonyl iron, which is taken up by phagocytes, to eliminate neutrophils. However, this also depletes up to two-thirds of the mononuclear phagocytic cells that are necessary for antigen presentation in PBMC cultures (Krakowka and Ringler 1985).

A preliminary investigation was therefore undertaken to compare PBMC yield and responses to mitogen and specific antigens after density centrifugation with and without carbonyl iron treatment.

5.2 Materials and Methods

5.2.1 PBMC isolation and culture

Atopic (n=16) and healthy dogs (n=16) were recruited according to the criteria described in Chapter 2.1. Blood samples were collected as described in Chapter 2.2.2. Each sample was diluted 1:1 with sterile citrated PBS. Half of each diluted sample was incubated with 20mg/ml carbonyl iron (Sigma) in an Erlenmeyer flask with gentle mixing at 37°C for one hour. The other half was stored at 4°C.

PBMCs were isolated from each sample and cultured as previously described (Chapter 2.6). $1x10^5$ cells were cultured in 200µl volumes with tenfold dilutions of ConA from 0.0005-50µg/ml and 1/10-1/100,000 dilutions of *Bordetella bronchiseptica* (Intrac®), canine parvovirus (Nobivac ParvoC®), *Leptospira* (Nobivac L®) and mixed canine distemper, parainfluenza, adenovirus and parvovirus (DHPPi) (Nobivac DHPPi®) vaccine antigens. Duplicate plates were cultured for three and five days (ConA) or five and seven days (vaccine antigens).

5.2.2 Data analysis

Cell proliferation was assessed as previously described (Chapter 2.6.2.3). PBMC proliferation was expressed as a delta count; i.e. (mean cpm per test wells – mean cpm background wells) – (mean cpm resting wells – mean cpm background wells).

Unpaired t-tests with Welch's correction for unequal variance (Instat®) were used to compare the PBMC yield and proliferation between atopic and healthy blood samples incubated with and without carbonyl iron.

5.3 Results

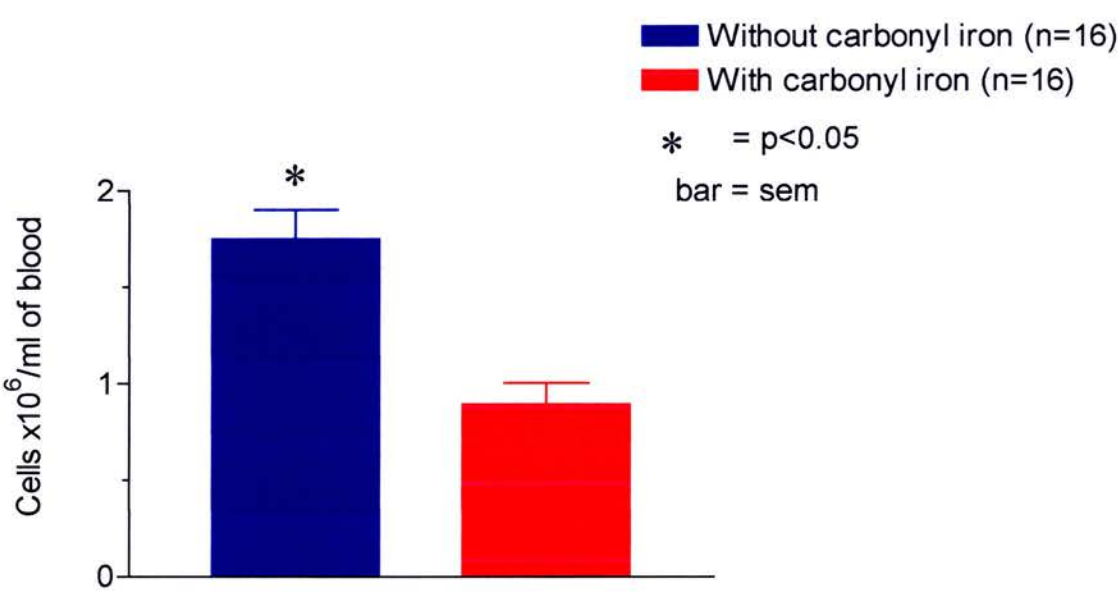
5.3.1 Overall cell yield and viability

The total cell yield per ml of blood varied from 0.72 - 2.5 x10^6 cells/ml of blood for samples without carbonyl iron and from 0.3 - 1.82 x10^6 cells/ml for those incubated with carbonyl iron. Significantly fewer cells were isolated by density centrifugation after incubation with carbonyl iron ($p<0.05$) (Table 5.1 and Figure 5.1). In all cases the viability of the isolated cells exceeded 95%.

Table 5.1 – Cell yields after PBMC isolation from whole blood

Mean (SD) yield x10^6 cells/ml blood	
Without carbonyl iron	With carbonyl iron (n=16)
1.75 (0.60)	0.89 (0.44)

Figure 5.1 – Total number of PBMCs isolated with and without prior incubation with carbonyl iron



5.3.2 PBMC yield

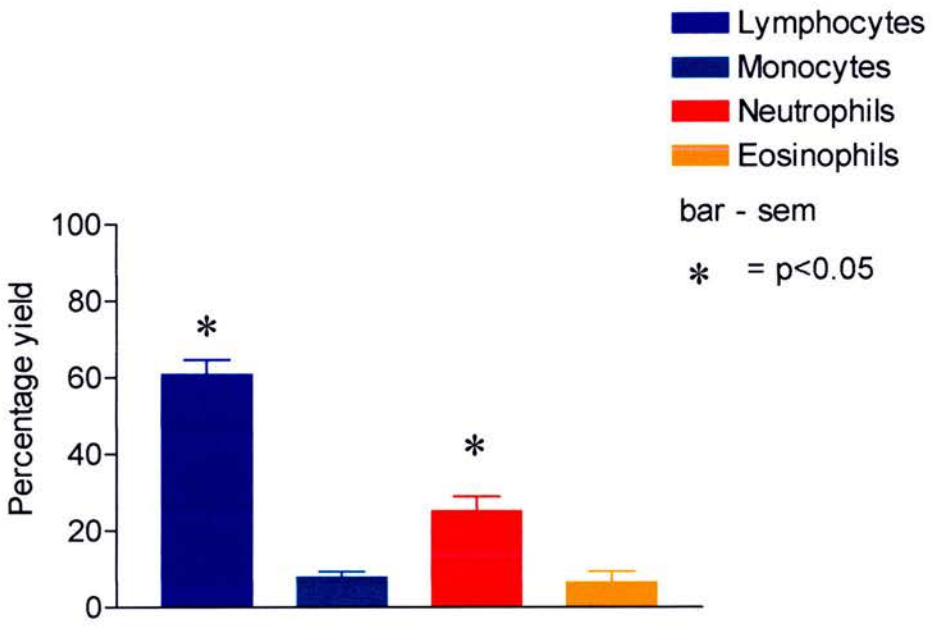
Prior incubation of whole blood with carbonyl iron significantly increased the proportion of lymphocytes and decreased contamination with neutrophils in the PBMC populations ($p<0.05$) (Table 5.2 and Figure 5.2). The percentage yields of monocytes and eosinophils were also reduced, although the differences were not significant. However, the mean number of lymphocytes and monocytes significantly declined from 1.2 to 0.79 x 10^6 cells/ml of blood after treatment with carbonyl iron ($p<0.05$).

Table 5.2 – PBMC cell population with and without prior incubation with carbonyl iron

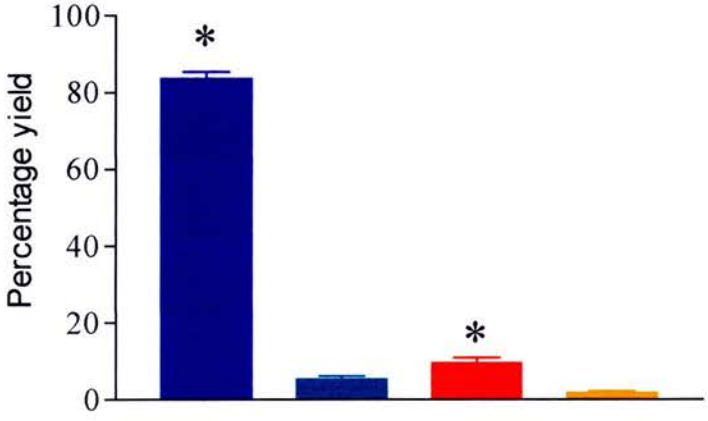
	Mean (SD) percentage of PBMCs			
	Lymphocytes	Monocytes	Neutrophils	Eosinophils
Without carbonyl iron (n=16)	60.7 (15.6)	7.9 (5.7)	25.1 (15.4)	6.4 (12.1)
With carbonyl iron (n=16)	83.5 (7.5)	5.4 (3.4)	9.4 (5.6)	1.7 (2.2)

Figure 5.2 – PBMC populations isolated from whole blood by density centrifugation over 1.077g/L ficoll

a – Without prior incubation with carbonyl iron



b – With prior incubation with carbonyl iron

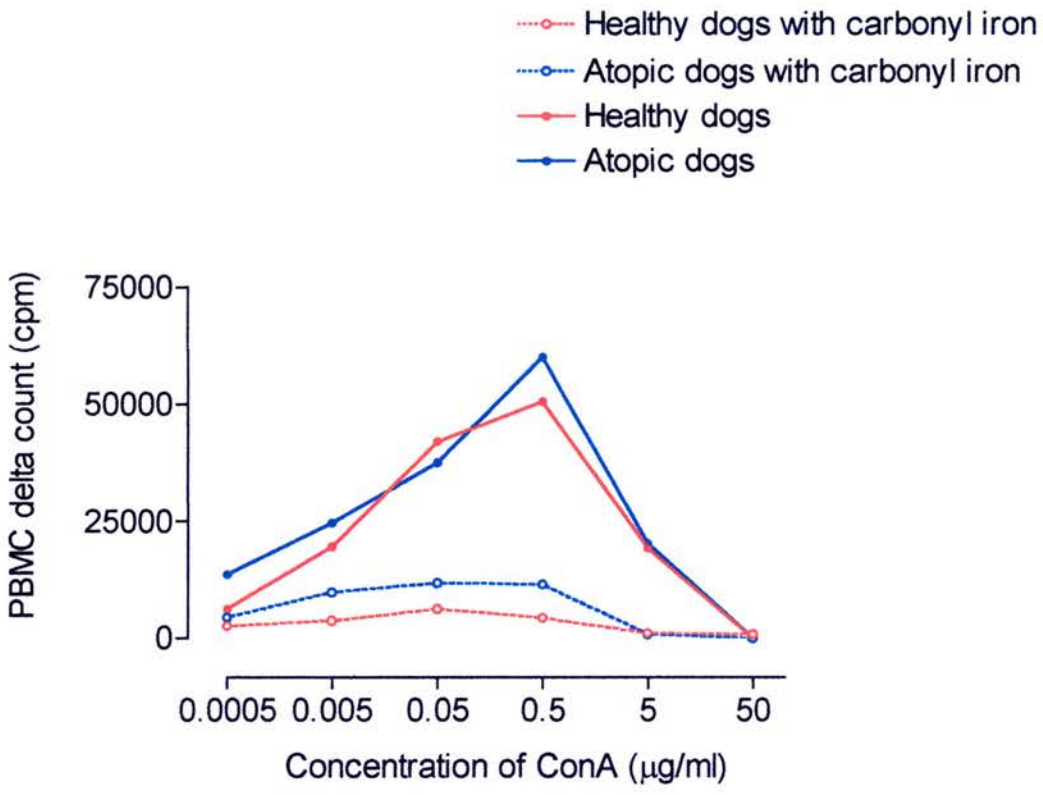


5.3.3 PBMC responses to ConA

Dose response curves to ConA were established for PBMC cultures at three and five days (Table 5.3a and b; Figure 5.3a and b). At each concentration the PBMCs isolated after incubation with carbonyl iron exhibited less proliferation than the corresponding PBMCs isolated without carbonyl iron. Optimum responses were seen with 0.5μg/ml ConA. At this concentration PBMCs isolated without carbonyl iron had a significantly greater response at both days three and five compared to those isolated with carbonyl iron ($p<0.05$). There were, however, no significant differences in the response to ConA between PBMC cultures from atopic and healthy dogs, or between three and five day cultures.

Figure 5.3 – Responses to ConA in PBMC cultures isolated with and without prior incubation with carbonyl iron

a – Day three of culture



b – Day five of culture

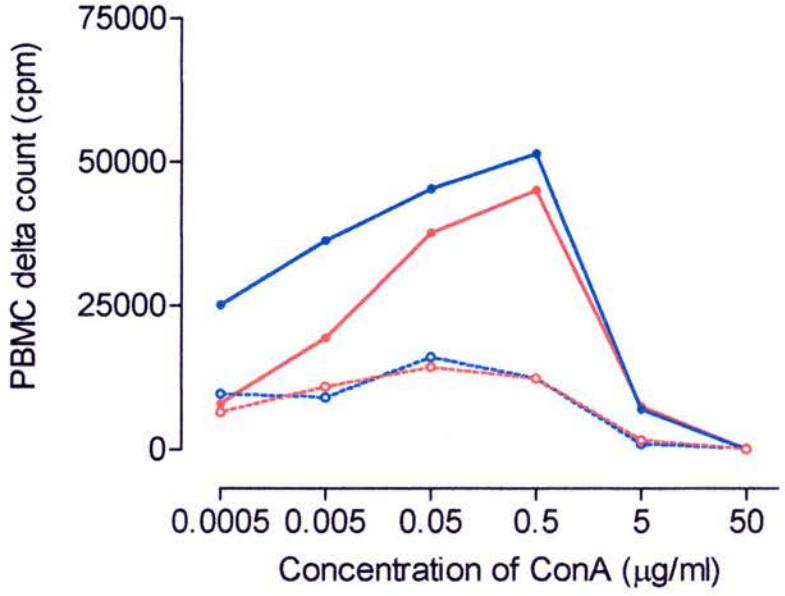


Table 5.3 – PBMC responses to ConA

a – Day three of culture

ConA (μg/ml)	Mean (SD) PBMC delta counts (cpm)			
	With carbonyl iron		Without carbonyl iron	
	Atopic dogs (n=7)	Healthy dogs (n=7)	Atopic dogs (n=8)	Healthy dogs (n=8)
50.0	0 (0)	804 (2266)	46 (112)	13 (21)
5.0	858 (1648)	1077 (1803)	20269 (23426)	19175 (8854)
0.5	11482 (7150)	4338 (3462)	60041 (26494)	50558 (16683)
0.05	11753 (8657)	6220 (2880)	37511 (24478)	41997 (15458)
0.005	9854 (5510)	3728 (3492)	24673 (19294)	19622 (5918)
0.0005	4506 (2901)	2592 (2846)	13641 (15821)	6291 (3099)

b – Day five of culture

ConA (μg/ml)	Mean (SD) PBMC delta counts (cpm)			
	With carbonyl iron		Without carbonyl iron	
	Atopic dogs (n=6)	Healthy dogs (n=7)	Atopic dogs (n=6)	Healthy dogs (n=6)
50.0	82 (218)	30 (38)	1 (4)	18 (49)
5.0	941 (1529)	1570 (2603)	7409 (5135)	7006 (6530)
0.5	12327 (8222)	12266 (8851)	45057 (23196)	51432 (31635)
0.05	15974 (4940)	14236 (10141)	37650 (18530)	45329 (33574)
0.005	8974 (8271)	10858 (11253)	19351 (23305)	36298 (37062)
0.0005	9589 (10934)	6419 (7228)	7929 (2890)	25156 (39440)

5.3.4 PBMC responses to vaccine antigens

Vaccine antigen specific PBMC proliferation was assessed at days five and seven of culture. The response to all the vaccines at day five was very low (Table 5.4a-d; Figure 5.4 a-d). Furthermore, there were no significant differences between atopic and healthy PBMCs or PBMCs isolated with and without carbonyl iron.

Proliferation after seven days of culture was much higher for all the vaccine extracts (Table 5.5a-d; Figure 5.5a-d). Nevertheless, the response to the *Leptospira* vaccine was still very poor, whether or not the PBMCs had been isolated after carbonyl iron incubation. For the other vaccines, the proliferative response of PBMCs isolated without carbonyl iron was higher than for PBMCs isolated with carbonyl iron. Optimum proliferation was seen with a 1/100,000 dilution of DHPPi, a 1/10,000 dilution of canine parvovirus and a 1/10 dilution of *B. bronchiseptica*. At these concentrations, the proliferation of PBMCs isolated without carbonyl iron was significantly greater than PBMCs isolated with carbonyl iron for parvovirus and *B. bronchiseptica* ($p<0.05$), but not for DHPPi. The PBMC response to all the vaccines at day seven was very variable between individuals, some of which did not show any responses to one or more of the vaccines. There were, however, no significant differences in the responses between PBMCs isolated from atopic or healthy dogs.

Table 5.4 – PBMC responses to vaccine antigens at day five of culture

a – DHPPi

Vaccine dilution	Mean (SD) PBMC delta counts (cpm)			
	With carbonyl iron		Without carbonyl iron	
	Atopic dogs (n=5)	Healthy dogs (n=5)	Atopic dogs (n=5)	Healthy dogs (n=6)
1/10	80 (179)	8 (19)	0 (0)	143 (200)
1/100	75 (106)	23 (51)	0 (0)	809 (1614)
1/1000	193 (240)	158 (354)	452 (887)	530 (1077)
1/10,000	336 (290)	475 (632)	538 (1078)	464 (585)
1/100,000	461 (391)	415 (362)	988 (995)	43 (67)

b – Parvovirus

Vaccine dilution	Mean (SD) PBMC delta counts (cpm)			
	With carbonyl iron		Without carbonyl iron	
	Atopic dogs (n=5)	Healthy dogs (n=5)	Atopic dogs (n=5)	Healthy dogs (n=6)
1/10	63 (141)	9 (21)	28 (68)	156 (143)
1/100	654 (1429)	358 (498)	336 (588)	225 (292)
1/1000	582 (692)	91 (203)	735 (1215)	127 (473)
1/10,000	953 (911)	336 (559)	262 (304)	1069 (1549)
1/100,000	702 (308)	197 (277)	560 (749)	1224 (1676)

Table 5.4 contd. – PBMC responses to vaccine antigens at day five of culture

c – *B.bronchiseptica*

Vaccine dilution	Mean (SD) PBMC delta counts (cpm)			
	With carbonyl iron		Without carbonyl iron	
	Atopic dogs (n=5)	Healthy dogs (n=5)	Atopic dogs (n=5)	Healthy dogs (n=6)
1/10	669 (803)	67 (71)	930 (765)	872 (1476)
1/100	451 (507)	75 (59)	762 (2853)	1758 (1371)
1/1000	413 (528)	133 (173)	1558 (664)	513 (1856)
1/10,000	369 (553)	150 (210)	789 (667)	383 (818)
1/100,000	543 (701)	104 (222)	341 (977)	482 (302)

d - *Leptospira*

Vaccine dilution	Mean (SD) PBMC delta counts (cpm)			
	With carbonyl iron		Without carbonyl iron	
	Atopic dogs (n=5)	Healthy dogs (n=5)	Atopic dogs (n=5)	Healthy dogs (n=6)
1/10	0 (0)	0 (0)	0 (0)	47 (106)
1/100	0 (0)	0 (0)	0 (0)	10 (23)
1/1000	0 (0)	0 (0)	0 (0)	4 (8)
1/10,000	13 (31)	60 (134)	0 (0)	81 (157)
1/100,000	449 (503)	138 (257)	0 (0)	227 (349)

Table 5.5 – PBMC responses to vaccine antigens at day seven of culture

a – DHPPi

Vaccine dilution	Mean (SD) PBMC delta counts (cpm)			
	With carbonyl iron		Without carbonyl iron	
	Atopic dogs (n=6)	Healthy dogs (n=5)	Atopic dogs (n=8)	Healthy dogs (n=8)
1/10	0 (0)	0 (0)	6 (16)	61 (173)
1/100	0 (0)	6 (13)	129 (366)	45 (76)
1/1000	161 (336)	73 (163)	624 (956)	268 (335)
1/10,000	925 (1337)	62 (136)	2395 (2910)	1766 (2452)
1/100,000	1687 (2524)	0 (0)	2801 (2901)	3644 (5063)

b – Parvovirus

Vaccine dilution	Mean (SD) PBMC delta counts (cpm)			
	With carbonyl iron		Without carbonyl iron	
	Atopic dogs (n=6)	Healthy dogs (n=7)	Atopic dogs (n=8)	Healthy dogs (n=8)
1/10	745 (1190)	28 (67)	0 (0)	198 (378)
1/100	725 (610)	220 (382)	553 (1484)	673 (1081)
1/1000	659 (1006)	295 (378)	1699 (1860)	2161 (2485)
1/10,000	857 (1179)	520 (821)	4043 (3980)	3668 (3872)
1/100,000	1453 (2935)	259 (335)	3616 (2524)	3126 (3549)

Table 5.5 contd. – PBMC responses to vaccine antigens at day seven of culture

c – *B. bronchiseptica*

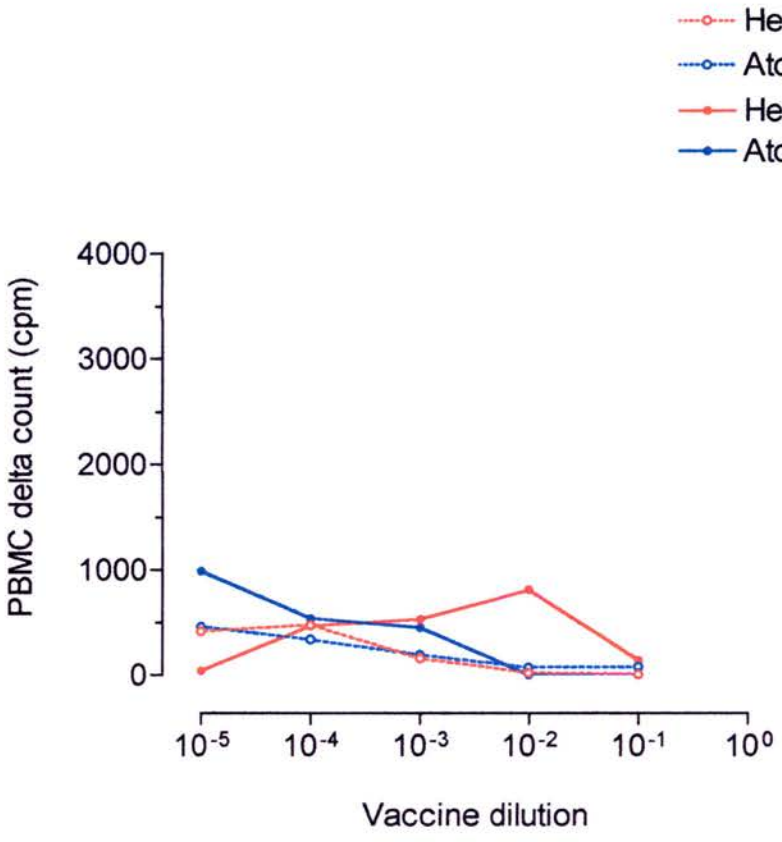
Vaccine dilution	Mean (SD) PBMC delta counts (cpm)			
	With carbonyl iron		Without carbonyl iron	
	Atopic dogs (n=6)	Healthy dogs (n=7)	Atopic dogs (n=8)	Healthy dogs (n=8)
1/10	234 (380)	196 (441)	5080 (3426)	6025 (8676)
1/100	877 (1731)	209 (489)	4387 (3968)	7199 (13752)
1/1000	122 (163)	219 (513)	3932 (3921)	2547 (4581)
1/10,000	30 (46)	473 (1048)	1095 (1474)	1388 (1949)
1/100,000	8 (18)	66 (141)	1811 (1952)	681 (966)

d – *Leptospira*

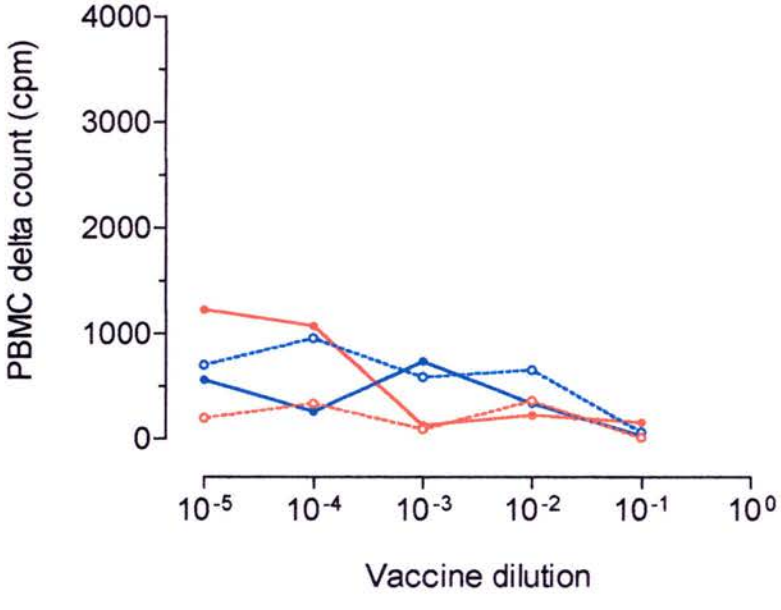
Vaccine dilution	Mean (SD) PBMC delta counts (cpm)			
	With carbonyl iron		Without carbonyl iron	
	Atopic dogs (n=6)	Healthy dogs (n=6)	Atopic dogs (n=8)	Healthy dogs (n=7)
1/10	0 (0)	0 (0)	0 (0)	0 (0)
1/100	0 (0)	133 (326)	0 (0)	0 (0)
1/1000	0 (0)	0 (0)	0 (0)	133 (353)
1/10,000	0 (0)	0 (0)	1691 (4270)	319 (652)
1/100,000	366 (594)	19 (46)	2298 (2575)	887 (1027)

Figure 5.4 – PBMC responses to vaccine antigens at day five of culture

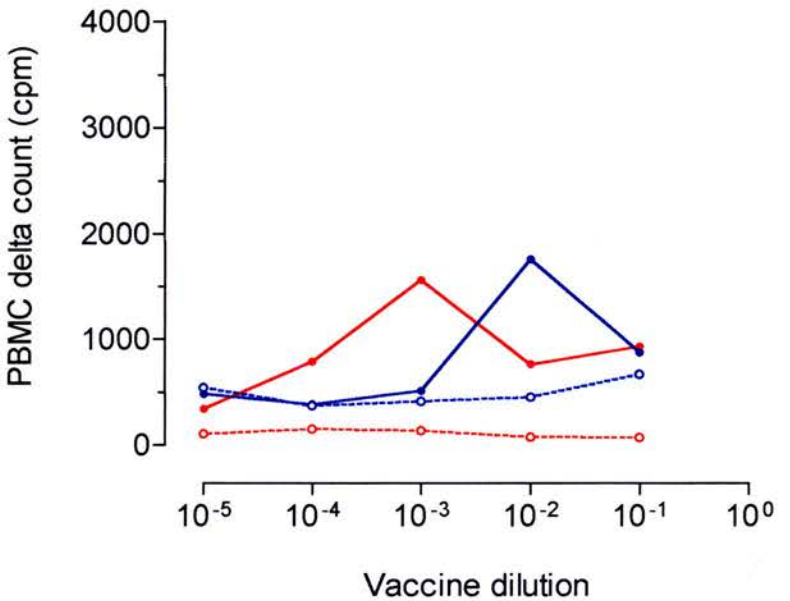
a – DHPPi



b – Parvovirus



c – *B. bronchiseptica*



d - *Leptospira*

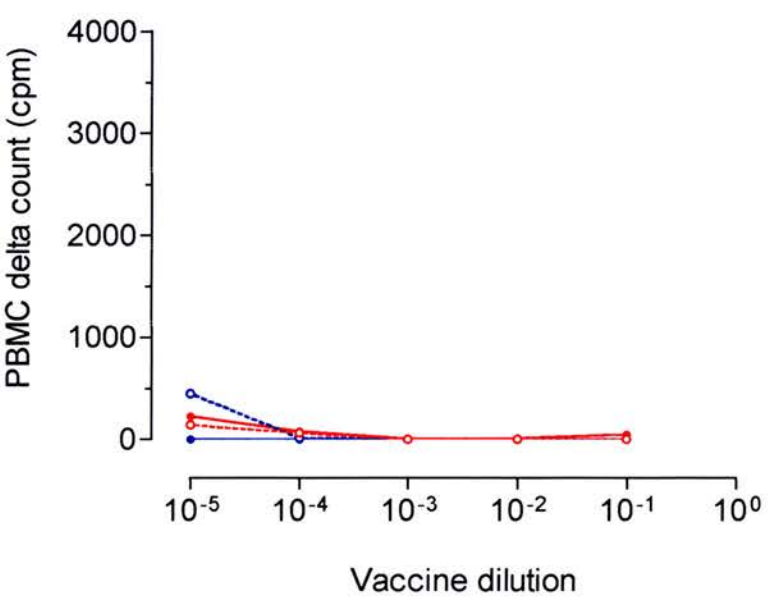
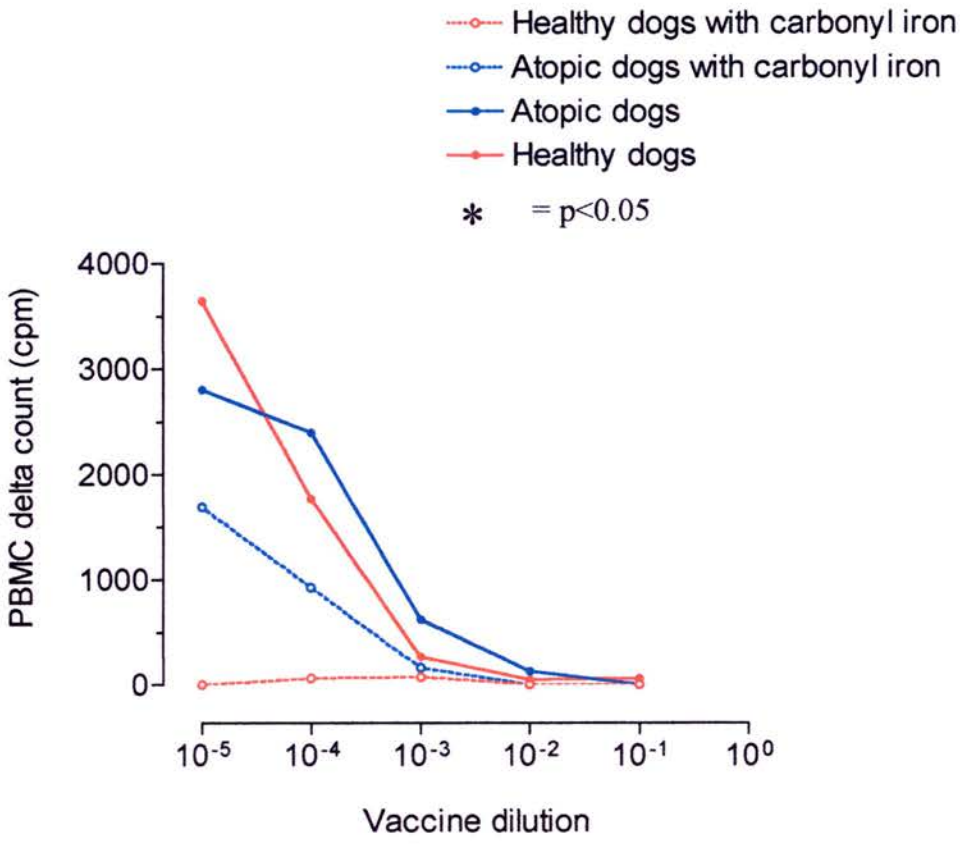
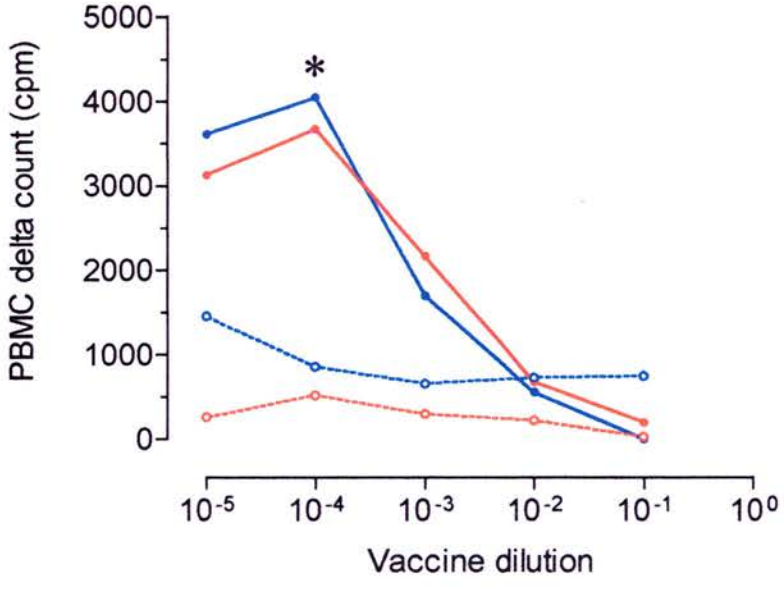


Figure 5.5 – PBMC responses to vaccine antigens at day seven of culture

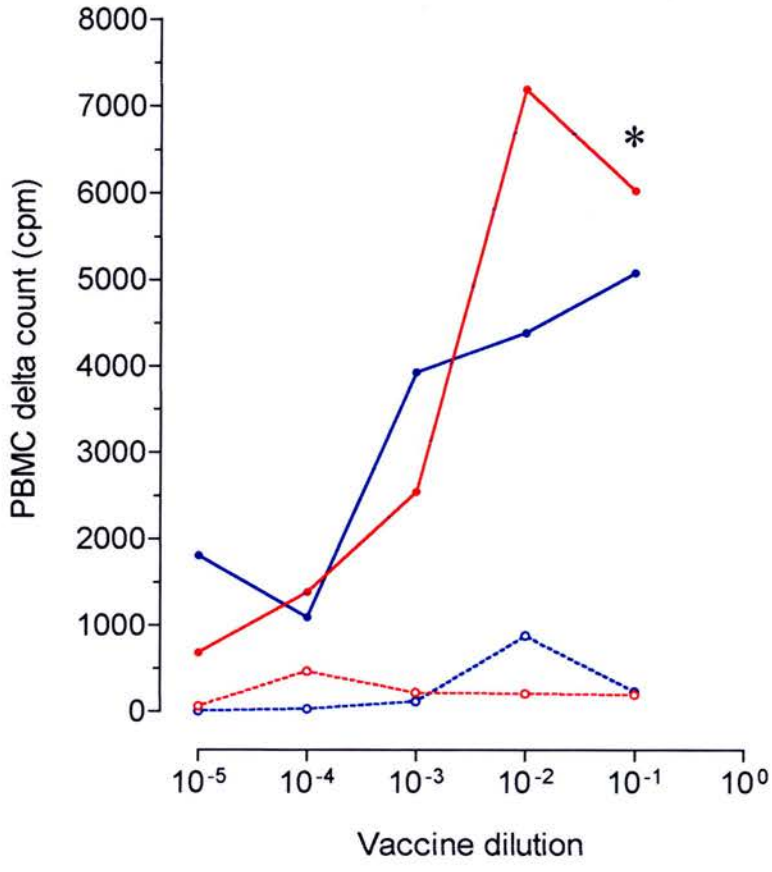
a – DHPPi



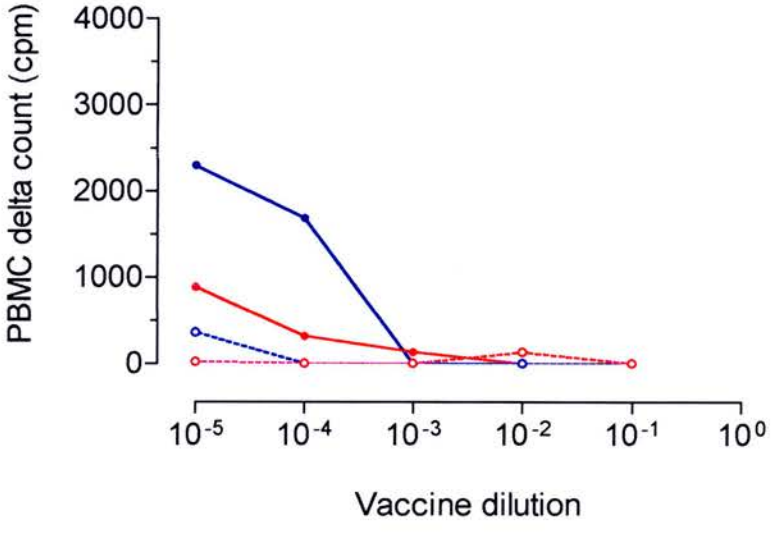
b – Parvovirus



c – *B.bronchiseptica*



d – *Leptospira*



5.4 Discussion

5.4.1 PBMC responses to ConA

Variable responses to mitogens have been previously reported in canine PBMC cultures (Helfand and others 1992), but all the PBMC cultures in this study exhibited robust responses to ConA. This stimulates T-cell proliferation in a manner that closely mimics antigen stimulation, and is used to demonstrate that PBMCs are capable of *in vitro* proliferation (Lydyard and Grossi 1998). There were no significant differences between the dose response curves to ConA established for PBMCs isolated from atopic and healthy dogs at both three and five days. This shows that PBMCs isolated from both groups of dogs behave in similarly *in vitro*. Any differences in later experiments, therefore, are likely to be allergen specific.

5.4.2 PBMC responses to vaccine antigens

The vaccine antigens were used to demonstrate that the PBMC cultures were capable of an antigen specific recall response *in vitro*. Only limited PBMC proliferation was seen after five days of culture. Proliferation after seven days, however, was much greater, with marked differences emerging between PBMCs isolated with and without carbonyl iron. Significantly greater responses to *B. bronchiseptica* and parvovirus were seen in the PBMCs isolated without carbonyl iron in both healthy and atopic dogs. This indicates these antigens are capable of stimulating a recall response. Although the responses were quite variable between individual dogs, these findings further indicate that PBMCs isolated from atopic and healthy dogs are capable of quantitatively similar antigen specific responses *in vitro*. In contrast, responses to *Leptospira* were still low after seven days and responses to DHPPi were highly

No significant differences, furthermore, were observed in the response to these two antigens between any of the groups of PBMCs.

Both atopic and healthy dogs are likely to have been exposed to these antigens. *Leptospira*, parvovirus and DHPPi are commonly included in canine vaccination protocols. Vaccination with a live attenuated strain of *B. bronchiseptica* is commonly performed prior to entry into boarding kennels. Most of the atopic and healthy dogs in this study had received at least one primary course of vaccination, but it was not always clear whether they had received booster vaccinations or been vaccinated against *B. bronchiseptica* according to the data sheet recommendations. Natural exposure to *B. bronchiseptica*, adenovirus 1 and parainfluenza (in DHPPi) is common in domestic and kennelled dogs, but natural and vaccine immunity wanes after 6-12 months (Ford and Vaden 1990). Infection with distemper and parvovirus is relatively uncommon, but naturally acquired and vaccine immunity is robust for at least 12 months (Hoskins 1990). However, distemper virus has been reported to inhibit canine PBMC proliferation *in vitro* (Kristensen and others 1982b). *Leptospira* and DHPPi, therefore, are probably unsuitable as recall stimuli. A previous study using vaccine antigens in canine PBMC cultures also reported a variable response (Corato and others 1997). This may be due to sub-optimal culture conditions or cytopathic effects, although the cultures in that and the present study that did respond did so very well. No reliable and universal recall antigen has been established in dogs and the effects of vaccination and natural exposure on the cellular responses to these antigens are unclear.

5.4.3 The effect of incubation with carbonyl iron on PBMC yield and culture

Prior incubation with carbonyl iron significantly reduced PBMC responses to both the non-specific mitogen ConA and specific vaccine antigens, making this technique unsuitable for studying canine PBMC proliferation. This may be due to depletion of phagocytic monocytes, which are necessary for PBMC proliferative responses to mitogens or antigens *in vitro* (Hogenesch and Felsburg 1989). B-cells may also act as APCs in culture, but at least 4% monocytes are considered necessary for *in vitro* PBMC proliferation (Letwin and Quimby 1987). The mean yield in this study after carbonyl iron treatment was 5.4%, which is close to this minimum figure. In addition, ingestion of particulate iron, or trace amounts carried over from the washing steps, could inhibit *in vitro* antigen presentation and subsequent cell proliferation. These findings are in contrast to a previous study where prior incubation with carbonyl iron increased the PBMC response to ConA (Krakowka and Ringler 1985). However, although the response to ConA in PBMCs isolated with carbonyl iron was similar to that in the present study, the response in PBMCs isolated without carbonyl iron was only 20% of that reported here.

In line with previous findings (Letwin and Quimby 1987; Rivas and others 1995), density centrifugation over 1.077g/L ficoll gave rise to a degree of granulocyte contamination. However, as terminally differentiated cells these shouldn't contribute to cell proliferation. Release of cytokines and other mediators could affect PBMC responses, but whole blood stimulation assays have been used to investigate cell mediated immunity in canine leishmaniosis (Martínez-Moreno and others 1995) and there is no significant difference in proliferation or cytokine production between whole blood and PBMC responses to mitogens in humans (Yaqoob and others 1999). Other

authors have advocated the use of 62.5% Percoll® (Amersham-Pharmacia Biotech, Amersham, UK) (Wagner and others 1999) (S.C. Bell, University of Liverpool, personal communication). However, in the author's hands this technique led to very poor cell yields (<2.0 x10^5 cells/ml of blood) and viability (<75%) and was not explored further.

Cell yields using both techniques were considerably lower than circulating leucocyte populations in dogs (6-15 x10^6/ml; reference range, Royal [Dick] School of Veterinary Studies Clinical Laboratory, University of Edinburgh), suggesting a considerable number of cells are lost during density centrifugation over 1.077g/L ficoll. Furthermore, the number of cells isolated after carbonyl iron incubation was 0.89 x10^6/ml. This is lower than the reference range for lymphocytes (1.0-6.0 x10^6/ml), suggesting that there is also loss of mononuclear cells with this technique. Nevertheless, the viability of the isolated cells exceeded 95% and density centrifugation appears to be a reliable technique for the isolation of viable canine PBMCs.

5.4.4 Conclusion

This study has shown that incubation of whole blood with carbonyl iron reduces neutrophil contamination of PBMC populations, but as it also inhibits *in vitro* proliferative responses, is not suitable for investigating PBMC proliferation. ConA is an appropriate non-specific stimulus to ensure that canine PBMCs are capable of *in vitro* proliferation, whereas canine parvovirus and *B. bronchiseptica* can be used to assess their ability to mount an antigen specific recall response. Antigen specific responses appear to require seven days of culture using these protocols.

6. Peripheral blood mononuclear cell responses to *Dermatophagoides* species allergens in canine atopic dermatitis

6.1 Introduction

Canine atopic dermatitis has been viewed as an IgE/mast cell mediated disease (Scott and others 2001a). However, IgE production is dependent on 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 and others 1997) and humans (Leung 2000). Several studies in humans have demonstrated PBMC proliferation to crude *Dermatophagoides* extracts and purified allergens (Leung 2000). In one unpublished study, PBMCs isolated from dogs with atopic dermatitis proliferated in response to both a crude *D. pteronyssinus* extract and the 98/104kD major allergen, but not Der p 1 or Der p 2 (Shaw 2000).

The aim of this study 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. The study also compared the responses of PBMCs from dogs with atopic dermatitis to group 1 and 2 allergens and the 98/104kD major allergen purified from *D. farinae,* as well as to crude extracts of *D. farinae*, *D. pteronyssinus* and *D. microceras*.

6.2 Materials and Methods

6.2.1 Isolation of the 98/104kD major allergen from *D. farinae*

The 98/104kD *D. farinae* major allergen (discussed in Chapter 4.4) was purified from the crude Greer *D. farinae* extract by high-pressure liquid chromotography (Waters 486/600S/626 system; Waters-Millipore, Macclesfield, UK) carried out by A.D. Pemberton, The University of Edinburgh Department of Veterinary Clinical Studies. Twenty fractions were collected into sterile PBS and separated by SDS-PAGE using individual wells (Chapter 2.4). Western blots of these gels were probed with a single positive serum sample and imaged as described in Chapter 2.5. The 98/104kD fraction was diluted to 1mg/ml in sterile PBS and stored at –20°C as described in Chapter 2.3.

6.2.2 PBMC isolation and culture

Atopic (n=30) and healthy dogs (n=10) were recruited according to the criteria described in Chapter 2.1. Blood samples were collected as described in Chapter 2.2.2. PBMCs were isolated from each sample and cultured as previously described (Chapter 2.6). $1x10^5$ cells were cultured in 200µl volumes with tenfold dilutions of the Greer *D. farinae* extract from 500-0.05µg/ml. In the second part of the study, $1x10^5$ cells were cultured in 200µl volumes containing 50µg/ml of the Greer *D. farinae*, Greer *D. pteronyssinus* and ALK *D. microceras* extracts, and 5.0µg/ml of the ALK Der f 1, ALK Der f 2 and the 98/104kD major allergen purified from the Greer *D. farinae*. Positive control wells with 0.5µg/ml ConA, 1/10 dilution of *B. bronchiseptica* (Intrac®) and 1/10,000 dilution of canine parvovirus (Parvo C®) were included in

each plate. $1x10^5$ cells in 200µl of medium and wells with 200µl medium only were included as negative controls and background respectively (Chapters 2.6 and 5.4).

6.2.3 Data analysis

Cell proliferation was assessed as previously described (Chapter 2.6.2.3). PBMC proliferation was expressed as: (mean cpm per test wells – mean cpm background wells) – (mean cpm resting wells – mean cpm background wells). One-way analyses of variance with Tukey's post tests were used to compare cell proliferation between groups (Instat®). Significance was set at $p<0.05$.

6.3 Results

6.3.1 Intradermal tests (IDT)

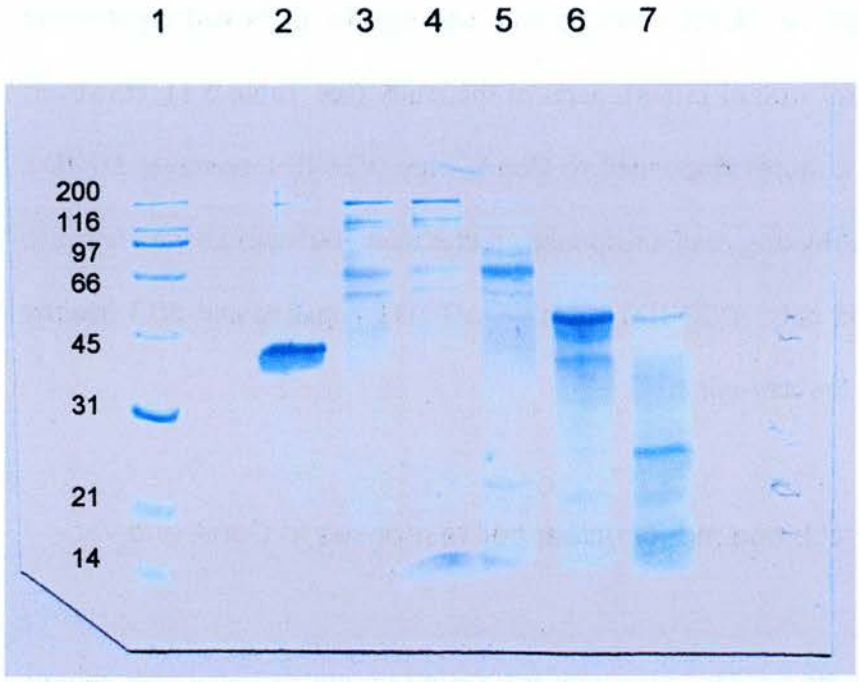
For the first part of the study, dogs with a clinical diagnosis of atopic dermatitis were divided into those with a positive IDT for *D. farinae* (n=10), and those with a negative IDT (n=7). In the second set of experiments all of the 13 atopic dogs had a positive IDT for *D. farinae* and *D. pteronyssinus*. Individual responses were occasionally seen to variety of other mite, insect, epidermal, pollen and mould allergens, although none were noted in the IDT negative group.

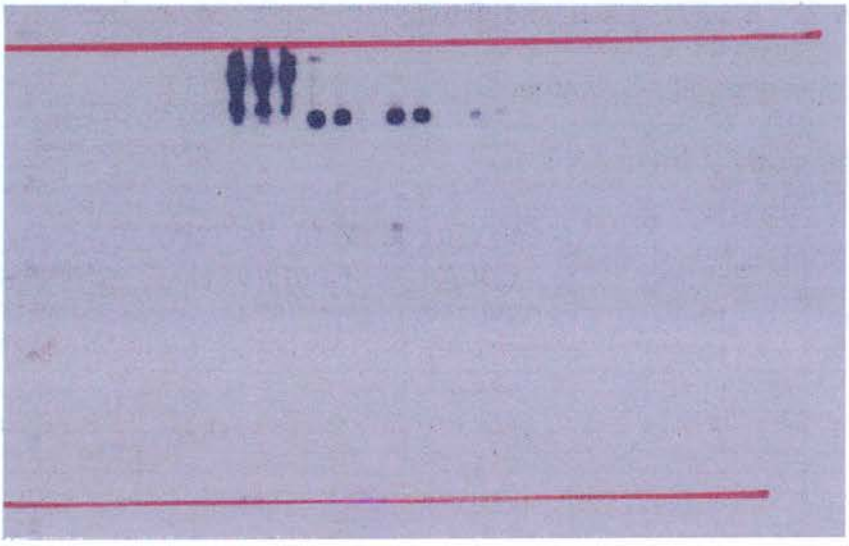
6.3.2 Purification of the 98/104kD major allergen from *D. farinae*

On the basis of the protein migration patterns after SDS-PAGE, it appeared that the 98/104kD major allergen co-purified in two out of the 20 fractions with a 128kD protein. Western blots with a single strongly positive serum sample, however, revealed that the IgE binding to these fractions was specific for the 98/104kD major allergen (Figure 6.1).

Figure 6.1 - Purification of the 98/104kD major allergen from *D. farinae*

a - Western blot (reducing buffer)





b - Immunoblot (non-reducing buffer)

Lane 1 - molecular weight markers (kD; Biorad broad range); lane 2 - fraction 1; lane 3 - fraction 2; lane 4 - fraction 3; lane 5 - fraction 4; lane 6 - fraction 5; lane 7 - fraction 6. Coomassie blue stain.

6.3.3 PBMC yield and responses to control stimuli

The protocols used in this study yielded a mean population of 83% PBMCs with greater than 95% viability. PBMCs from atopic and healthy dogs had comparable responses to the control stimuli in both parts of the study (see Table 6.1). However, whilst all the PBMC cultures responded to ConA, only 9/23 IDT positive, 3/7 IDT negative and 6/13 healthy dogs had a response greater than 1000cpm above resting to canine parvovirus, and only 10/23 IDT positive, 6/7 IDT negative and 8/13 healthy dogs responded to *B. bronchiseptica*.

Table 6.1 - Peripheral blood mononuclear cell responses to ConA and vaccine antigens

DF - *Dermatophagoides farinae*; sd - standard deviation; cpm - counts per minute (for conA, *Bordetella bronchiseptica* and Parvovirus expressed as [test wells-background] minus [resting wells-background]).

a – Part one of the study (Chapter 6.3.4)

	Mean (sd) delta counts (cpm)			
	Resting	ConA	*Bordetella*	Parvovirus
DF positive atopic dogs (n=10)	1909 (1848)	49935 (23178)	5736 (6535)	3975 (4485)
DF 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)

b – Part two of the study (Chapters 6.3.5 and 6.3.6)

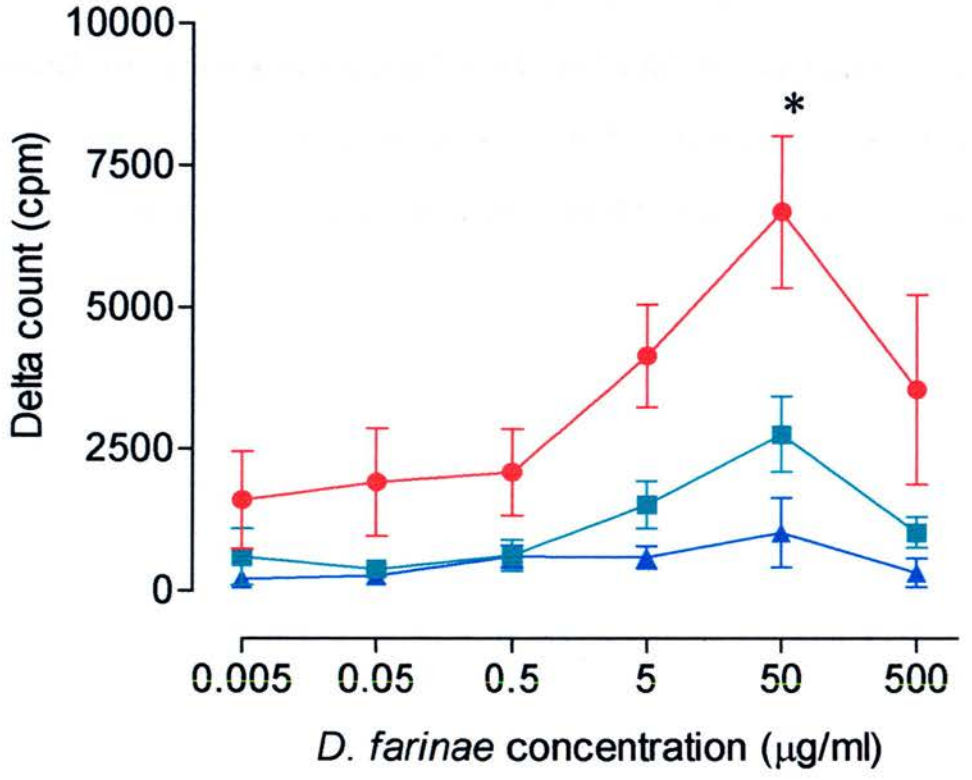
	Mean (sd) delta counts (cpm)			
	Resting	ConA	*Bordetella*	Parvovirus
Dogs with atopic dermatitis (n=13)	2562 (1618)	49571 (20307)	1295 (3823)	2013 (2799)
Healthy dogs (n=3)	2101 (2013)	27354 (12235)	168 (291)	0 (0)

6.3.4 PBMC responses to *D. farinae* in atopic and healthy dogs

Dose response curves to *D. farinae* were established for PBMCs isolated from atopic dogs with positive IDT reactions, atopic dogs with negative IDT reactions and healthy dogs. PBMCs from the IDT positive atopic dogs (n=10) showed greater proliferation to all concentrations of the *D. farinae* extract than did PBMCs from either IDT negative atopic dogs (n=7) or healthy dogs (n=10) (Figure 6.2). Optimum proliferation was seen with 50μg/ml *D. farinae*. At this concentration there was significantly greater proliferation of PBMCs from the IDT positive dogs with atopic dermatitis than either the IDT negative dogs with atopic dermatitis or healthy dogs ($p<0.05$). There was no significant difference between the latter two groups.

Figure 6.2 – PBMC responses to *D. farinae* in atopic and healthy dogs

● - Atopic dogs with positive IDT reactions to *D.farinae* (n=10); ■ - atopic dogs with negative IDT reactions (n=7); ▲ - healthy dogs (n=10); cpm – counts per minute (expressed as [mean cpm test wells – background] – [mean cpm resting wells – background]); bar – sem; * - $p<0.05$



6.3.5 PBMC responses to *D. farinae*, *D. pteronyssinus* and *D. microceras* in dogs with atopic dermatitis

There was significantly greater proliferation in the PBMC cultures from dogs with atopic dermatitis (n=13) to the crude extracts of *D. farinae* and *D. pteronyssinus* than to the crude extract of *D. microceras* ($p<0.05$). There were, however, no significant differences between the responses to *D. farinae* and *D. pteronyssinus* (Table 6.2 and Figure 6.3). None of the control PBMCs isolated from three healthy dogs responded to any of the crude *Dermatophagoides* extracts.

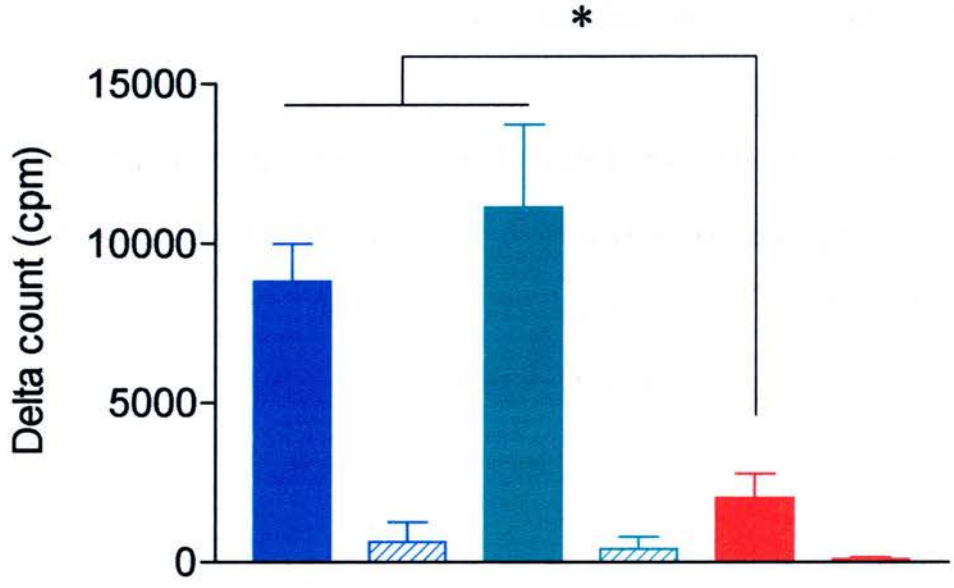
Table 6.2 – *In vitro* responses of PBMCs isolated from dogs with atopic dermatitis and healthy dogs to crude and purified *Dermatophagoides* allergens

sd – standard deviation; cpm – counts per minute (expressed as [mean cpm test wells – background] – [mean cpm resting wells – background]).

Allergen	Mean delta counts (cpm) (sd; number of dogs)	
	Atopic dermatitis	Healthy dogs
Dermatophagoides farinae	9288 (3926; 13)	630 (1091; 3)
D. pteronyssinus	11111 (9309; 13)	418 (632; 3)
D. microceras	2003 (2767; 13)	77 (132; 3)
98/104kD *D. farinae* allergen	5517 (2945; 7)	0 (0; 3)
Der f 1	1570 (1422; 11)	36 (62; 3)
Der f 2	252 (325; 11)	0 (0; 3)

Figure 6.3 – PBMC responses to *D. farinae*, *D. pteronyssinus* and *D. microceras* dogs with atopic dermatitis (n=13) and healthy dogs (n=3)

■ - *D. farinae*; ■ - *D. pteronyssinus*; ■ - *D. microceras*; cpm – counts per minute expressed as (mean test wells – background) – (mean resting wells – background); Solid bars - atopic dogs; hatched bars - healthy dogs; bar – sem; * - $p<0.05$.

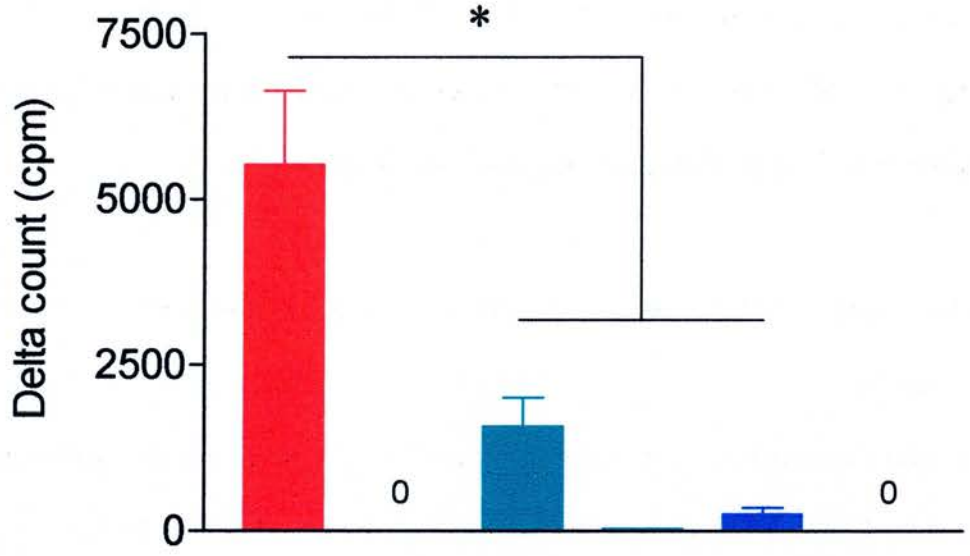


6.3.6 PBMC responses to major and minor *D. farinae* allergens in dogs with atopic dermatitis

When PBMCs isolated from dogs with atopic dermatitis dogs were incubated with purified *D. farinae* extracts, there was significantly greater response to the 98/104kD allergen than to either Der f 1 or Der f 2 ($p<0.05$). There was no significant difference between the responses to Der f 1 and Der f 2 (Table 6.2 and Figure 6.4). None of the PBMCs isolated from three healthy dogs reacted to the purified allergens.

Figure 6.4 – PBMC responses to major and minor allergens purified from *D. farinae*

■ - 98/104kD major allergen (atopic dogs n=7; healthy dogs n=3); ■ - Der f 1 (atopic dogs n=11; healthy dogs n=3); ■ - Der f 2 (atopic dogs n=11; healthy dogs n=3); Solid bars - atopic dogs; hatched bars - healthy dogs; cpm – counts per minute, expressed as (mean test wells – background) – (mean resting wells – background); bar – sem; * - $p<0.05$.



6.4 Discussion

6.4.1 PBMC responses to *D. farinae* in canine atopic dermatitis

To the author's knowledge these results document for the first time the presence of circulating *D. farinae* specific T-cells in canine atopic dermatitis. PBMC responses to a crude *D. farinae* extract under optimum conditions were significantly greater in atopic dogs with positive IDT reactions to *D. farinae* than in either IDT negative atopic dogs or healthy dogs. This suggests that circulating T-cell responses to *D. farinae* are greater in sensitised dogs, supporting a role for allergen specific T-cells in the pathogenesis of canine atopic dermatitis. Furthermore, the association of *D. farinae* specific PBMC proliferation with skin test reactivity implies that these cells are supporting specific IgE production (Lydyard and Grossi 1998).

6.4.2 PBMC responses to major and minor *D. farinae* allergens in canine atopic dermatitis

This study also demonstrated that memory T-cells in canine atopic dermatitis are specific to the 98/104kD allergen purified from *D. farinae*, rather than to Der f 1 or Der f 2. This is in contrast to humans, where T-cells from 80% or more of atopic patients will respond to group 1 and 2 allergens (Thomas and others 1998a), but similar to a study in which T-cells from atopic dogs responded to a 98kD allergen purified from *D. pteronyssinus*, but not Der p 1 or Der p 2 (Shaw 2000). Immunoblotting studies have also shown that the 98/104kD protein is a major *Dermatophagoides* allergen in canine atopic dermatitis, whereas group 1 and group 2 proteins appear to be minor allergens (Noli and others 1996; McCall and others 2000; Nuttall and others

2001a). Possible explanations for the variation in major and minor allergens between canine and human atopic dermatitis were discussed in Chapter 4.4.

Canine T-cell responses appear, therefore, to strongly correlate with IgE levels, suggesting that they support specific IgE production. This, however, is not necessarily the case in humans, where the frequency of T-cell responses to the minor allergen Der p 7 are comparable with those to the major allergen Der p 1 (Thomas and others 1999). The authors of this study speculate that Der p 7 might act in an IgE independent manner.

6.4.3 Comparative PBMC responses to *D. farinae*, *D. pteronyssinus* and *D. microceras*

PBMCs isolated from dogs sensitised to *D. farinae* and *D. pteronyssinus* showed significantly greater responses to the crude *D. farinae* and *D. pteronyssinus* extracts, than to the crude *D. microceras* extract. It was not possible to skin test the atopic dogs with the *D. microceras* extract, but an earlier study (Nuttall and others 2001a) demonstrated that canine serum IgE specific to *D. farinae* and *D. pteronyssinus* did not bind to western blots of *D. microceras*. These results suggest that both B- and T-cell epitopes are either specific to individual *D. farinae* and *D. pteronyssinus* allergens or to cross-reacting allergens present only in these two species, as discussed in Chapter 4.4.

It is widely accepted that *D. farinae* is a more important allergen to dogs, even though less common than *D. pteronyssinus* in the UK (Sture and others 1995). An Australian study in humans also found that T-cell responses to *D. farinae* matched those to *D. pteronyssinus* despite a similar paucity of *D. farinae* in the environment

(Thomas and others 1999). However, a recent survey in an area of Brazil thought to be low in *D. farinae* found equal levels of Der f 1 and Der p 1 in house dust (Sopelete and others 2000). Another study found that indoor Der f 1 and Der p 1 levels were only weakly correlated, and were highly variable both within and between geographical areas (Gross and others 2000). Interestingly, high levels of Der f 1 in this study were associated with the presence of a dog, raising the possibility of a specific relationship between these species. However, the apparent predominance of *D. farinae* in canine dermatology could reflect the allergen concentration in extracts widely used for IDTs and serology, as discussed in Chapter 4.4.

6.4.4 PBMC responses to *D. farinae* in IDT negative atopic dogs

A few *D. farinae* negative atopic dogs did have appreciable PBMC responses to *D. farinae.* This could have been due to false negative skin tests (due to poor technique, degraded allergens, anti-inflammatory medication etc.). This, however, is unlikely as these were performed by experienced dermatologists (R.E.W. Halliwell, K.L. Thoday, P.B. Hill, A.W. Carter, P. Forsythe and S. Colombo; Dermatology Service University of Edinburgh Hospital for Small Animals). Discordant responses could also be due to false positive PBMC proliferation assays, although cultures where proliferation of the resting cells was similar to the positive controls were discarded.

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. In one mouse model of atopic dermatitis, $CD8^+$ and $CD4^+$ T-cell infiltration could be induced in the absence of an IgE response (Spergel and others 1999). This suggests that allergen specific type IV hypersensitivity can develop in some individuals. Al-

lergen specific type IV responses, however, have also been associated with protective immunity in humans (Maggi 1998). Despite this, the distribution of clinical signs and epidermal focussing of Langerhans' cells and lymphocytes in canine atopic dermatitis are evidence of percutaneous allergen uptake (Olivry and others 1997). It is possible some dogs develop a type IV hypersensitivity reaction akin to contact dermatitis rather than an IgE response (Kalish and Askenase 1999). A recent report speculated that skin lesions in canine atopic dermatitis are triggered and maintained by allergen specific type IV inflammatory reactions, although IgE was considered important in initial sensitisation (Sinke and others 2002). Late phase inflammatory responses and chronic lesions in human atopic dermatitis are also dominated by T_{H1}-type cell-mediated reactions (Thepen and others 1996). It is unclear if these 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. The role of T_{H1} cells and cell-mediated immune responses in canine atopic dermatitis clearly needs further study.

6.4.5 PBMC responses to *D. farinae* in healthy dogs

There was very little response to *D. farinae* in PBMCs from healthy dogs, even though most individuals should be exposed to ubiquitous allergens such as house dust mites. A low level of reactivity is also reported in PBMCs from healthy humans (Horneff and others 1996). However, PBMC responses to Der p 1 have been detected in both atopic and healthy humans using serum free media (Upham and others 1995;

Kawamura and others 1998). This suggests that 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 TGFβ inhibit inflammatory responses in healthy individuals (Muraille and Leo 1998; Hoyne and others 2000; Koulis and Robinson 2000). These cells may also limit *in vitro* responses to allergens in PBMCs isolated from those subjects. The role of IL-10 and TGFβ in canine atopic dermatitis is explored in Chapter 7.

6.4.6 Responses to control stimuli and cell yield

It is unlikely that these results reflect differential mitogenic activity in the *Dermatophagoides* extracts, as PBMCs from healthy dogs did not respond to any of the extracts, despite comparable responses to ConA, canine parvovirus and *B. bronchiseptica*. Inhibition by any of the extracts is also unlikely as some PBMC cultures did respond and all are routinely used in other studies (G.Nordskov Hansen; ALK, Hørsholm, Denmark, personal communication). All the PBMC cultures in this study responded to ConA, which stimulates T-cell proliferation in a manner that closely mimics antigen stimulation (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. However, despite similar responses in all three groups, the majority of PBMC cultures responded poorly. This may be due to sub-

optimal culture conditions or cytopathic effects, although, as described in Chapter 5, the cultures that did respond did so very well. No reliable and universal recall antigen has been established in dogs (Corato and others 1997) and the effects of vaccination and natural exposure on PBMC responses are unclear.

It was not possible to phenotype the reactive cells in this study. 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 and others 1982a; Rivas and others 1995). Granulocytes are also frequent as canine PBMCs and granulocytes have very similar densities (Letwin and Quimby 1987) and are difficult to separate by density centrifugation (Chapter 5). PBMC cultures in this study included 5-25% granulocytes, although these cells shouldn't contribute to overall cell proliferation as discussed in Chapter 5.4.

Both B- and T-cells could contribute to cell proliferation, although B-cells require interaction with activated $CD4^+$ T_H cells (Lydyard and Grossi 1998). Most of the response to the *Dermatophagoides* allergens seen in this study should be due to $CD4^+$ cells, particularly as exogenous antigens are usually presented by APCs in the context of an MHCII molecule to $CD4^+$ cells (Kalish and Askenase 1999). However, exogenous antigens can also be presented in the context of MHCI molecule 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 $CD4^+$ cells in PBMC proliferation assays.

6.4.7 Conclusions

In conclusion, this study has shown that PBMC responses to *D. farinae* allergens are specific to *D. farinae* sensitised atopic dogs. The T-cell responses in this study have also confirmed serological data that a 98/104kD allergen, rather than Der f 1 or Der f 2, is the most important *D. farinae* allergen in canine atopic dermatitis. This strongly suggests 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} deviation and peripheral tolerance in atopic and healthy dogs. Studies of the allergenic nature of the 98/104kD protein will also further our understanding of the relationship between allergens and skin disease.

7. The expression of T-helper 1, T- helper 2 and immunosuppressive cytokine gene transcripts in canine atopic dermatitis

7.1 Introduction

Recent interest has focused on the role of $CD4^+$ T_{H1} and T_{H2} cells in human atopic dermatitis (Leung 2000). Several studies have detected T_{H2} polarisation in the skin and PBMCs of atopic humans (van der Heijden and others 1991; van Reijsen and others 1992; Neumann and others 1996; Koning and others 1997a; Koning and others 1997b) and mouse models (Spergel and others 1999; Vestergaard and others 1999). In dogs, a study using non-quantitative RT-PCR demonstrated a T_{H2}-type cytokine pattern in 25% of atopic and a T_{H1}-type pattern in 25% of healthy canine skin samples (Olivry and others 1999b).

T_{H1} cytokines inhibit T_{H2} differentiation (Mosmann and Coffman 1987). T_{H1}-type cells, however, are found in chronic human atopic lesions (Werfel and others 1996) and dominate atopy patch test sites after 48 hours (Thepen and others 1996), suggesting they also participate in the pathogenesis of chronic atopic dermatitis. Furthermore, healthy individuals do not develop T_{H1} induced cell-mediated inflammatory reactions to environmental allergens (Borish and Rosenwasser 1997). Instead, tolerance in healthy individuals may be mediated by the immunosuppressive cytokines IL-10 and TGFβ1 (Muraille and Leo 1998; Koulis and Robinson 2000).

Allergen induced activation of mast cells is widely believed to initiate inflammatory reactions in atopic skin (Welle 1997). SCF/*c-Kit* interactions enhance *in vitro* sur-

vival, proliferation, granularity, cytokine expression and degranulation of human (Brody and Metcalfe 1998) and canine (Brazís and others 2000) mast cells, although their importance in atopic dermatitis is unclear.

This study used semi-quantitative RT-PCR to compare the expression of the T_{H1}-type cytokines IFNγ, TNFα, IL-2 and IL-12, the T_{H2}-type cytokines IL-4 and IL-6, and the immunosuppressive cytokines IL-10 and TGFβ1, as well as SCF and *c-Kit* in atopic and healthy canine skin. The aim was to determine if canine atopic dermatitis is associated with polarised T_{H1}/T_{H2} responses as seen in humans.

7.2 Materials and Methods

7.2.1 Animals and samples

Twenty-eight dogs with atopic dermatitis and 16 healthy dogs were recruited according to the inclusion criteria described in Chapter 2.1. All the dogs with a clinical diagnosis of atopic dermatitis had an intradermal test with 56 allergens (Table 2.1) performed as described in Chapter 2.1.5.

Blood samples were collected from each dog as described in Chapter 2.2.2. Skin biopsies of non-lesional and lesional skin, where present, and control tissues were collected into RNA Later® from each healthy and atopic dog as described in Chapters 2.2.3 and 2.2.4 respectively.

7.2.2 PBMC cultures

PBMCs were isolated from whole blood as outlined in Chapter 2.6.2.1. The cells were cultured, collected into Tri-Reagent® and stored as described in Chapter 2.6.2.4.

7.2.3 Primer Design

Forward and reverse primers (Table 7.1) were designed from published canine sequences (Genbank®; accession numbers listed in Table 2.5) and generated as described in Chapter 2.7.2.1. Internal probes for each cytokine (Table 7.1) were generated and digoxigenin labelled as previously described (Chapter 2.7.2.2).

Table 7.1 - Cytokine primers sequences and optimum conditions for polymerase chain reactions

Cytokine	Primer sequence	Annealing temperature (°C)	$MgCl_2$ (mM)	pH	PCR Cycles	Product size (bp)
GAPDH	**Forward** - CCTTCATTGACCTCAACTACAT **Reverse** - CCAAAGTTGTCATGGATGACC **Internal** - CCCTCAAGATTGTCAGCAATGCC	55	2.0	8.6	35	400
IL-4	**Forward** - TAAAGGGTCTCACCTCCCAACTG **Reverse** - TAGAACAGGTCTTGTTTGCCATGC **Internal** - CACCAGCACCTTTGTCCACGGA	55	1.5	9.2	36	317
IL-6	**Forward** - CCACAAGCGCCTTCTCCCTGG **Reverse** - TCACGCACTCATCCTGCGACTG **Internal** - GAATCACTACCGGTCTTGTGGAG	60	1.0	8.3	35	532
IFNγ	**Forward** - TCGGACGGTGGGTCTCTTTTCG **Reverse** - CACTTTGATGAGTTCATTTATCGCC **Internal** - CAGCACCAGTAAGAGGGAGGAC	60	2.5	8.9	35	281
IL-2	**Forward** - CTCACAGTAACCTCAACTCCTGC **Reverse** - TTCTGTAATGGTTGCTGTCTCGTC **Internal** - ACACGCCCAAGAAGGCCACAGA	55	2.5	8.6	35	461
TNFα	**Forward** - ACTCTTCTGCCTGCTGCACTTTGG **Reverse** - GTTGACCTTTGTCTGGTAGGAGACGG **Internal** - CACCCACACCATCAGCCGCTTCGCCG	55	2.5	8.9	35	366
IL-12p35	**Forward** - AGCCTCACCGAGCCCAGGA **Reverse** - TAAGGCACAGGACCGTCATAAAAG **Internal** - TGGAGGCCTGCTTACCACTGGA	60	2.0	8.6	34	293
IL-12p40	**Forward** - GCCCCCGGAGAAATGGTGGTC **Reverse** - TTGTGGCACACGACCTTGGCTG **Internal** - ACCAGACCCACCCACAAACCTG	65	1.5	8.3	34	783

Table 7.1 contd. - Cytokine primers and optimum conditions for polymerase chain reactions

Cytokine	Primer sequence	Annealing temperature (°C)	$MgCl_2$ (mM)	pH	PCR Cycles	Product size (bp)
TGFβ1	**Forward** - AGTTAAAAGCGGAGCAGCATGTGG **Reverse** - GATCCTTGCGGAAGTCAATGTAGAGC **Internal** - GTTCAGTTCCAGCCCGCCGAGGT	55	2.5	9.2	35	434
IL-10	**Forward** - GTCCCTGCTGGAGGACTTTAAGA **Reverse** - TGGTCGGCTCTCCTACATCTCG **Internal** - AAGGCGGTGGAGCAGGTGAAGA	55	1.5	8.6	35	443
SCF	**Forward** - AGGGATCTGCGGGAAACGTGTG **Reverse** - TGCTGTCATTCCTAAGGGAGCTG **Internal** - CGGGATGGATGTTTTGCCTAGTC	55	1.0	8.6	35	519
c-Kit	**Forward** - GTCACCGTCTGGAAAACTAGTGG **Reverse** - AACAGTCATGGCCGCATCCGAC **Internal** - AACAAATCCATCCCCACACCCTG	55	1.0	9.2	36	462

7.2.4 RNA extraction and reverse transcription

RNA was extracted from the skin biopsies and control tissues as described in Chapter 2.7.3. RNA preparations with less than 120ng/µl RNA or 260nm:280nm absorbance ratios persistently less than 1.5 were discarded. The quality of each RNA preparation was assessed on a Northern gel (Chapter 2.7.3.2). RNA preparations from skin and control tissues were heparinased using the protocol described in Chapter 2.7.4. The heparinased (skin and positive control tissue) and non-heparinased (PBMCs) RNA samples were reverse transcribed and stored as described in Chapter 2.7.5.

7.2.5 PCR optimisation

PCRs were carried out in 50µl volumes as outlined in Chapter 2.7.6. The optimum $MgCl_2$ concentration, pH and annealing temperature for each primer pair were determined as follows. PCRs for each primer pair were carried out with positive control cDNA using the PCR buffers listed in Table 7.2. Identical PCR reactions were run for 35 cycles at annealing temperatures of 55°C, 60°C and 65°C. The PCR products were separated on 1.2% agarose gels and imaged as described in Chapter 2.7.7. The optimum reaction conditions (Table 7.1) were those yielding the greatest PCR product intensity and fidelity.

Table 7.2 - PCR optimisation buffers (PCR Optimisation Kit®; Roche)

Buffer	$MgCl_2$ (mM)	pH
A	1.0	8.3
B	1.5	8.3
C	2.0	8.3
D	2.5	8.3
E	1.0	8.6
F	1.5	8.6
G	2.0	8.6
H	2.5	8.6
I	1.0	8.9
J	1.5	8.9
K	2.0	8.9
L	2.5	8.9
M	1.0	9.2
N	1.5	9.2
O	2.0	9.2
P	2.5	9.2

The identity of each PCR product was confirmed by Southern blotting and hybridisation to digoxigenin labelled internal probes as described in Chapter 2.7.

To determine the optimum number of cycles for semi-quantitative PCR, 26 to 44 PCR cycles for each primer pair were performed using positive control cDNA under optimum conditions and the gels imaged as described. Standard curves were generated by plotting the number of PCR cycles against the log2 net band intensity of each PCR product (Figure 7.1). The optimum number of cycles (Table 7.1) for each cytokine primer pair was selected from the straight-line portion of the plot.

Figure 7.1 – Abundance of gene transcripts after 26 – 44 PCR cycles

Net band intensity = net intensity of fluorescence for each PCR product under 590nm ultra-violet light (Kodak IS440CF imaging system) and analysed using Kodak 1D software (see Chapter 2.7.7).

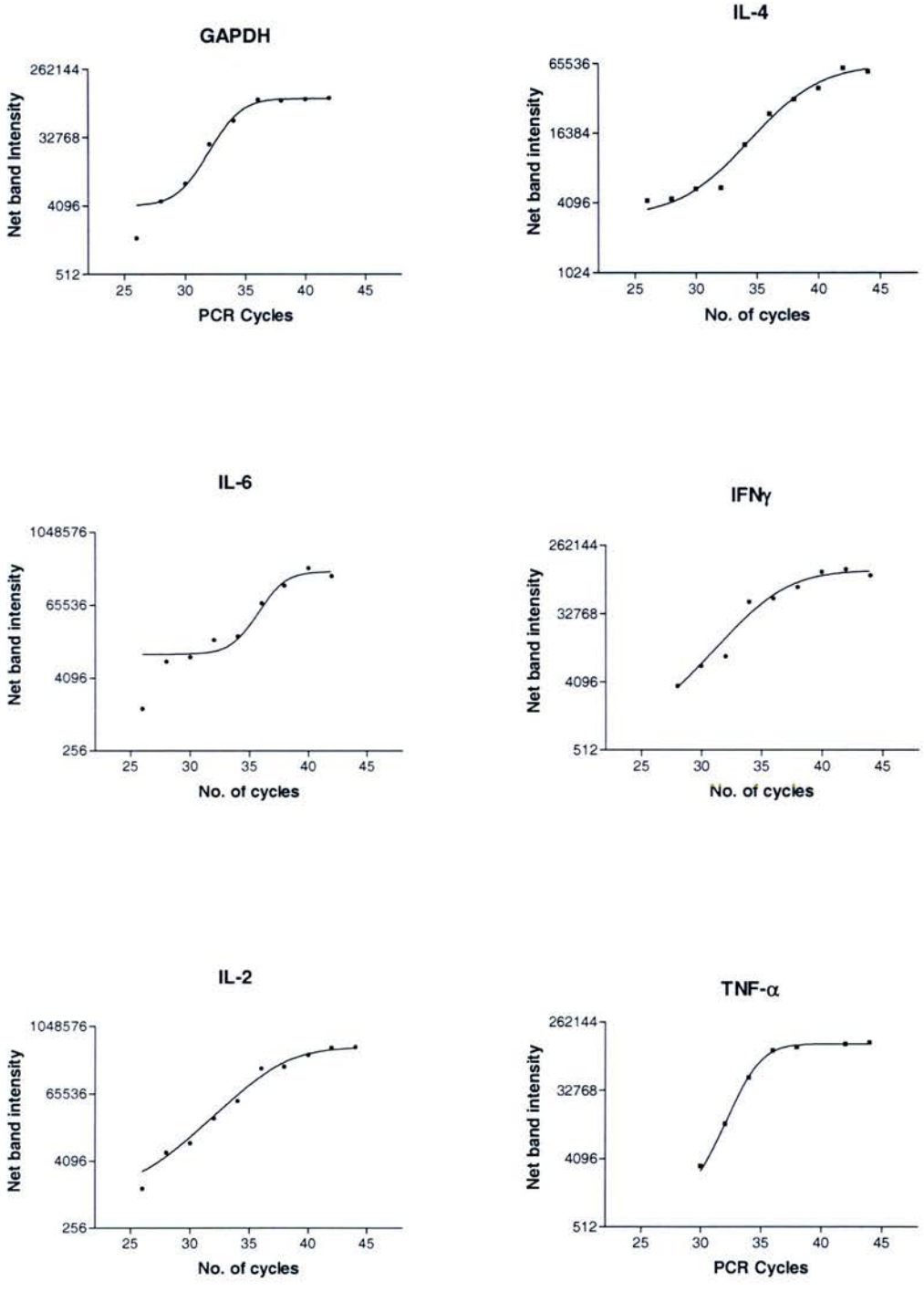
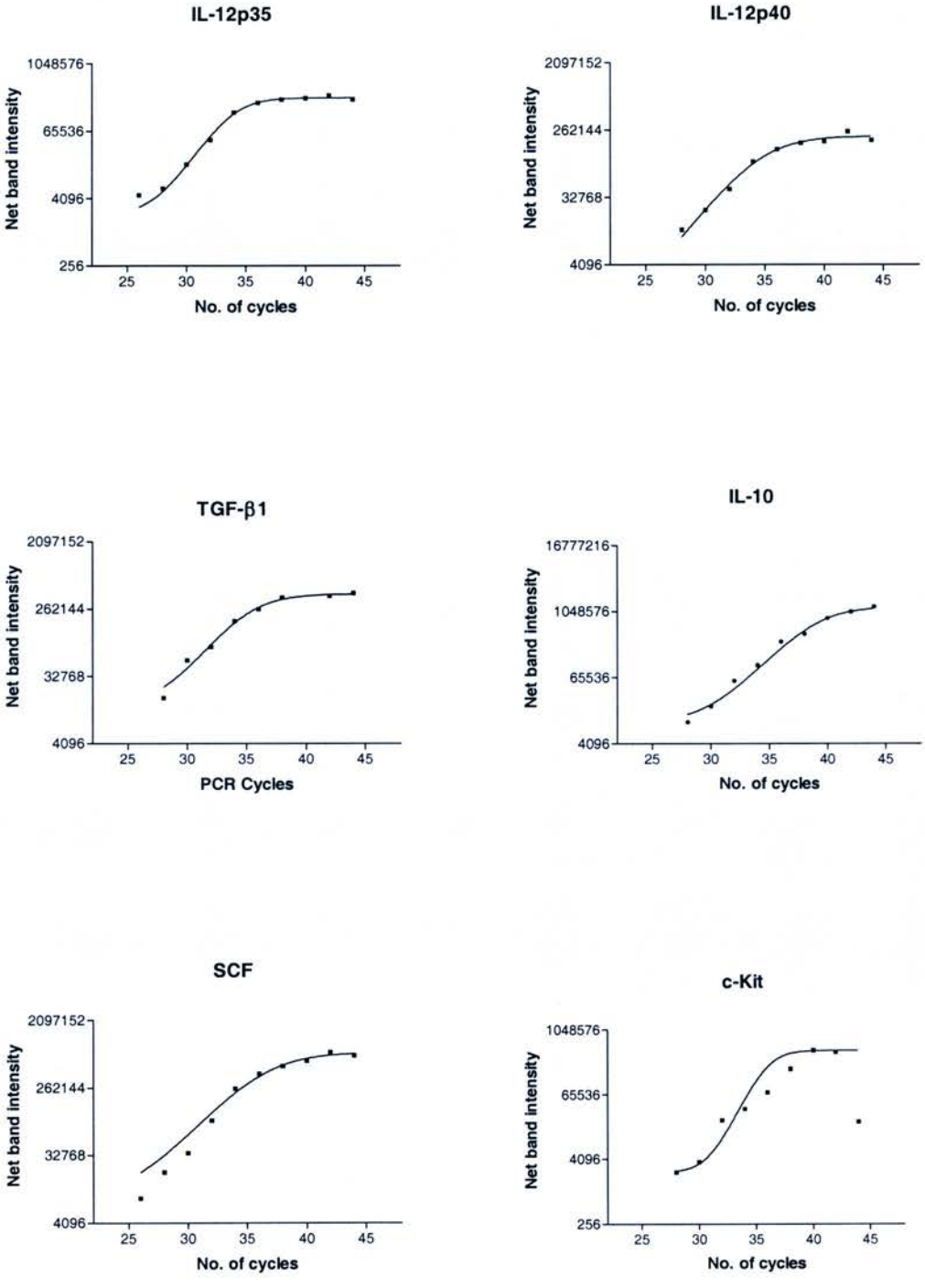


Figure 7.1 contd. – Abundance of gene transcripts after 26 – 44 PCR cycles



7.2.6 Selecting the optimum time point for cytokine gene expression in canine PBMCs cultures

The optimum time points for mRNA expression of each cytokine were determined by culturing PBMCs isolated from six atopic dogs with 0.5μg/ml ConA under optimum conditions for one, three, five and seven days. RNA was isolated from each culture, PCR reactions performed for each cytokine and the PCR products imaged as previously described. The level of expression of cytokine mRNA was determined by calculating net band intensity (cytokine)/net band intensity (GAPDH) for each sample. The optimum time point for mRNA expression of each cytokine was that yielding the greatest mean expression compared to GAPDH (Table 7.3 and Figure 7.2).

Table 7.3 Optimum length of PBMC cultures for isolation of cytokine mRNA

Cytokine	Optimum culture (days)
IL-4	5
IL-6	5
IFNγ	5
IL-2	5
TNFα	1
IL-12p35	5
IL-12p40	1
IL-10	1
TGFβ1	1
SCF	5
c-Kit	5

Figure 7.2 – Expression of cytokine gene transcripts by PBMCs cultured for 1 – 7 days

Relative intensity = cytokine band intensity/GAPDH band intensity (imaged under 590nm ultra-violet light Kodak IS440CF imaging system analysed using Kodak 1D software - Chapter 2.7.7); ● - number of cultures with quantifiable gene transcription (n = 6); ● - mean cytokine relative intensity; bar - standard error of the mean.

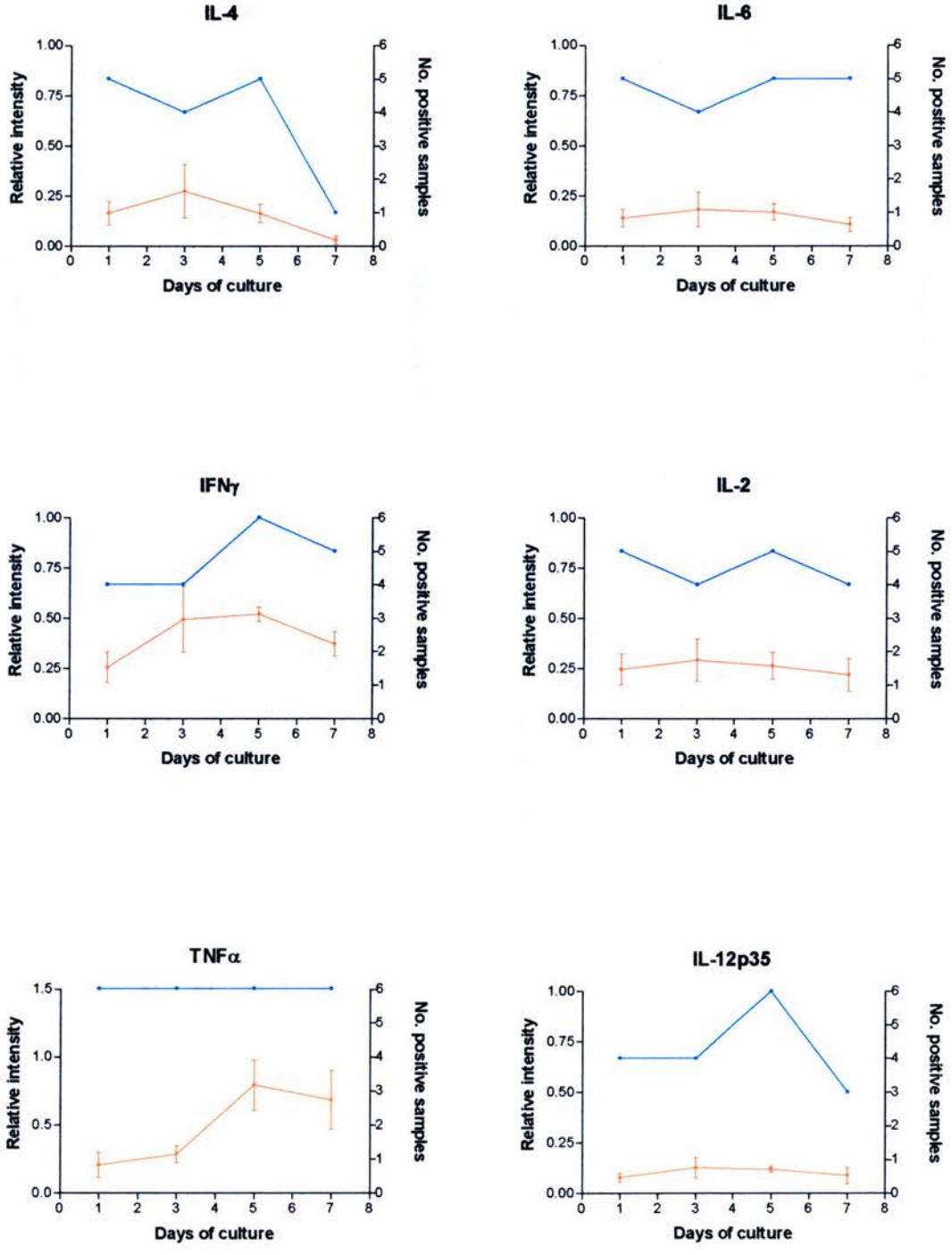
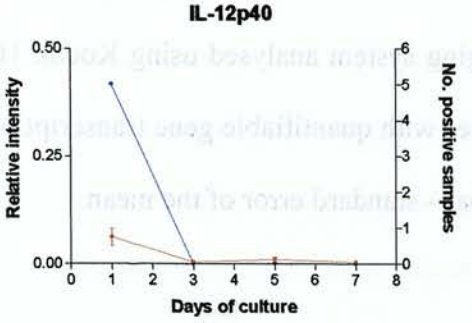
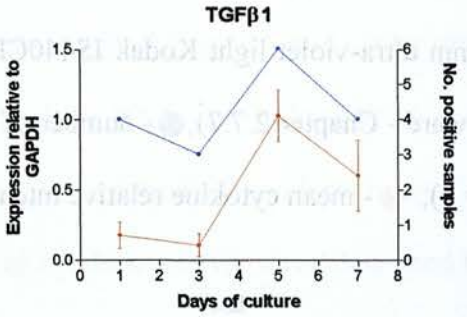
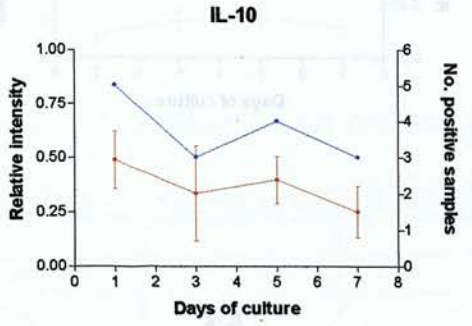
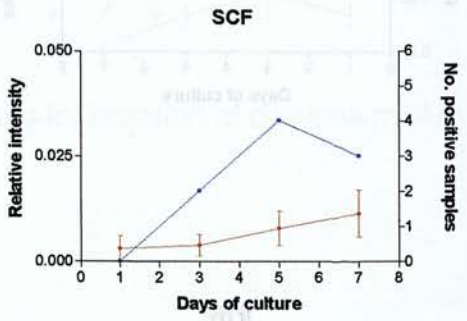


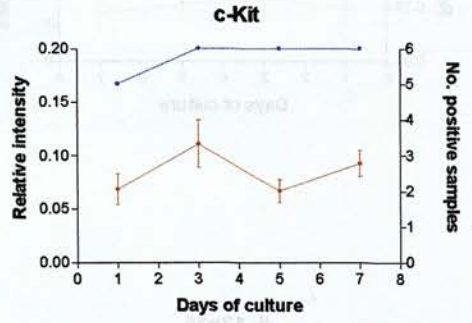
Figure 7.2 contd. – Expression of cytokine gene transcripts by PBMCs cultured for 1 – 7 days











7.2.7 Semi-quantitative PCR to determine the level of cytokine gene transcription in canine skin and PBMCs from atopic and healthy dogs

PCRs for each cytokine primer pair were performed with cDNA from each skin sample and PBMCs cultured for the appropriate length of time. Positive control cDNA samples and negative control wells (no DNA) were included in each PCR. PCR products were run on gels and imaged as before. Gels with no positive control signal or a negative control signal were discarded.

7.2.8 Data analysis

Cytokine expression in each sample was expressed as:

- Net band intensity (cytokine) / net band intensity (GAPDH)

After ensuring data were normally distributed one way analyses of variance with Tukey's post-tests (Instat®) were used to compare the level of cytokine gene transcrition in healthy, lesional atopic and non-lesional atopic skin, and between freshly isolated, mitogen and allergen stimulated PBMCs isolated from atopic and healthy dogs. The level of significance was set at $p<0.05$.

7.3 Results

7.3.1 Intradermal test reactivity

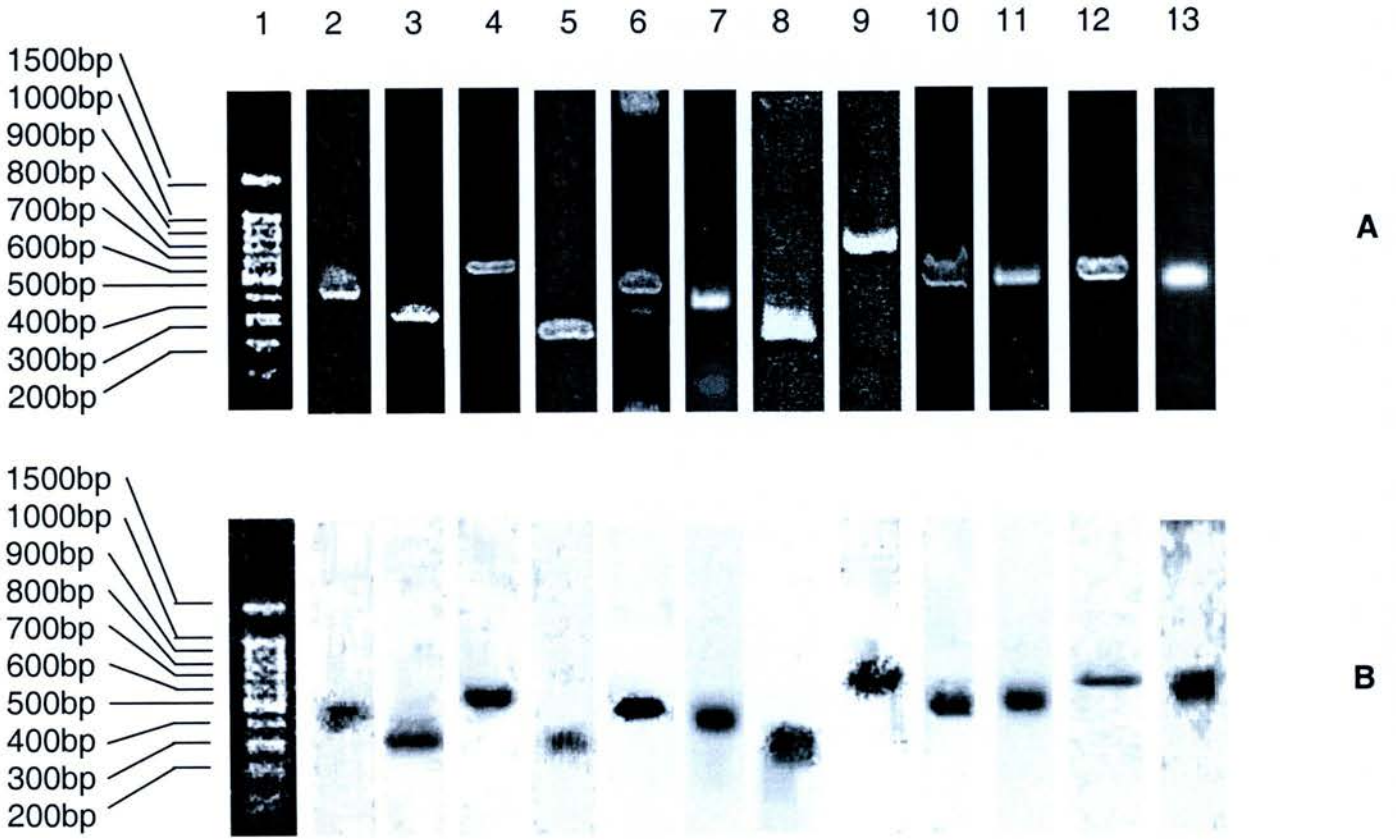
All the dogs with a clinical diagnosis of atopic dermatitis had positive skin tests to *D. farinae* and *D. pteronyssinus*. There were also a number of individual reactions to a range of other dust mites, epithelia, pollens and moulds.

7.3.2 PCR optimisation

The optimum reaction conditions established for each primer pair are detailed in Table 7.1. Single bands of the expected size for each cytokine were seen on the PCR gel and hybridisation blots using positive control material (Figure 7.3). This verified expression of mRNA for each cytokine in the positive control samples and confirmed primer specificity.

Figure 7.3 – Detection of cytokine gene transcription using positive control cDNA

A – 1.2% agarose gel with 0.5µg/ml ethidium bromide; B – Southern blotting and hybridisation to digoxigenin labelled internal probes; Lane 1 – 100bp DNA markers (Promega Corp.); Lane 2 – GAPDH; Lane 3 – IL-4; Lane 4 – IL-6; Lane 5 – IFNγ; Lane 6 – IL-2; Lane 7 – TNFα; Lane 8 – IL-12p35; Lane 9 – IL-12p40; Lane 10 – TGFβ1; Lane 11 – IL-10, Lane 12 – SCF; Lane 13 – *c-Kit*.



7.3.3 Cytokine gene transcription in atopic and healthy dogs

Variable levels of mRNA expression for all the cytokines were detected in each of the samples tested. Table 7.4 summarises the cytokine mRNA expression relative to GAPDH in lesional atopic, non-lesional atopic and healthy skin. Cytokine mRNA levels in freshly isolated, mitogen and allergen stimulated PBMCs from atopic and healthy dogs are summarised in Table 7.5.

Table 7.4 – Mean expression of cytokine mRNA relative to expression of GAPDH mRNA in canine skin (sd; number of samples)

	Lesional atopic dermatitis	Non-lesional atopic dermatitis	Healthy skin
T_{H1} cytokines			
IL-2	0.23 (0.17; 12)	0.07 (0.12; 22)	0.09 (0.05; 12)
IFNγ	0.24 (0.23; 12)	0.05 (0.05; 25)	0.07 (0.07; 12)
TNFα	2.16 (1.36; 8)	0.82 (0.58; 26)	0.58 (0.61; 10)
IL-12p35	0.18 (0.1; 12)	0.23 (0.29; 26)	0.28 (0.46; 12)
IL-12p40	0.59 (0.99; 12)	0.27 (0.57; 24)	0.67 (0.87; 12)
T_{H2} cytokines			
IL-4	0.89 (1.1; 9)	0.54 (0.87; 25)	0.03 (0.05; 10)
IL-6	0.25 (0.25; 12)	0.23 (0.33; 26)	0.11 (0.1; 12)
Immunosuppressive cytokines			
TGFβ1	0.46 (0.53; 8)	0.60 (0.39; 26)	1.07 (0.47; 12)
IL-10	0.66 (0.3; 12)	0.48 (0.42; 26)	0.41 (0.52; 12)
SCF	1.15 (1.08; 12)	1.46 (1.19; 26)	1.06 (0.63; 12)
c-Kit	0.61 (0.81; 8)	0.24 (0.26; 26)	0.12 (0.18; 11)

Table 7.5 – Mean expression of cytokine mRNA relative to expression of GAPDH mRNA in canine PBMCs (sd; number of samples)

Resting – freshly isolated; DF – cultured with 50μg/ml Greer *D. farinae* extract; ConA – cultured with 0.5μg/ml ConA.

PBMC culture	Resting		ConA		DF	
	Atopic	Healthy	Atopic	Healthy	Atopic	Healthy
T_{H1} cytokines						
IFNγ	0.3 (0.62; 13)	0.75 (0.93; 9)	1.23 (1.46; 8)	2.34 (0.96; 8)	1.69 (1.45; 10)	3.26 (1.43; 8)
IL-2	0.11 (0.15; 11)	0.32 (0.24; 8)	0.33 (0.47; 11)	1.03 (0.97; 8)	0.41 (0.67; 9)	0.99 (1.18; 7)
TNFα	0.92 (1.15; 11)	1.66 (1.44; 8)	3.02 (2.11; 5)	1.45 (1.02; 10)	3.26 (3.0; 9)	2.46 (2.27; 7)
IL-12p35	0.53 (0.54; 12)	0.29 (0.31; 8)	0.24 (0.3; 12)	0.73 (0.3; 8)	0.24 (0.23; 8)	0.91 (0.63; 7)
IL-12p40	0.13 (0.24; 11)	0.07 (0.12; 8)	0.05 (0.04; 9)	0.08 (0.06; 8)	0.18 (0.25; 8)	0.17 (0.19; 7)
T_{H2} cytokines						
IL-4	0.34 (0.52; 12)	0.09 (0.17; 8)	0.24 (0.3; 14)	0.43 (0.48; 8)	1.52 (1.67; 10)	0.08 (0.06; 9)
IL-6	0.04 (0.07; 14)	0.05 (0.07; 9)	0.29 (0.44; 13)	0.28 (0.23; 8)	0.18 (0.37; 8)	0.22 (0.18; 7)
Immunosuppressive cytokines						
IL-10	0.33 (0.23; 12)	0.42 (0.22; 8)	0.54 (0.40; 12)	1.07 90.57; 8)	0.46 (0.28; 8)	1.11 (0.38; 7)
TGFβ1	1.32 (1.01; 11)	1.75 (0.66; 8)	1.75 (1.0; 5)	2.0 (0.53; 10)	1.29 (0.69; 9)	2.8 (1.02; 7)
SCF	0.11 (0.15; 12)	0.04 (0.05; 6)	0.16 (0.28; 10)	0.09 (.017; 8)	0.21 (0.16; 11)	0.1 (0.13; 9)
c-Kit	0.01 (0.01; 12)	0.06 (0.03; 8)	0.08 (0.06; 11)	0.12 (0.12; 8)	0.08 (0.11; 7)	0.09 (0.07; 9)

7.3.3.1 Expression of T_{H2}-type cytokines

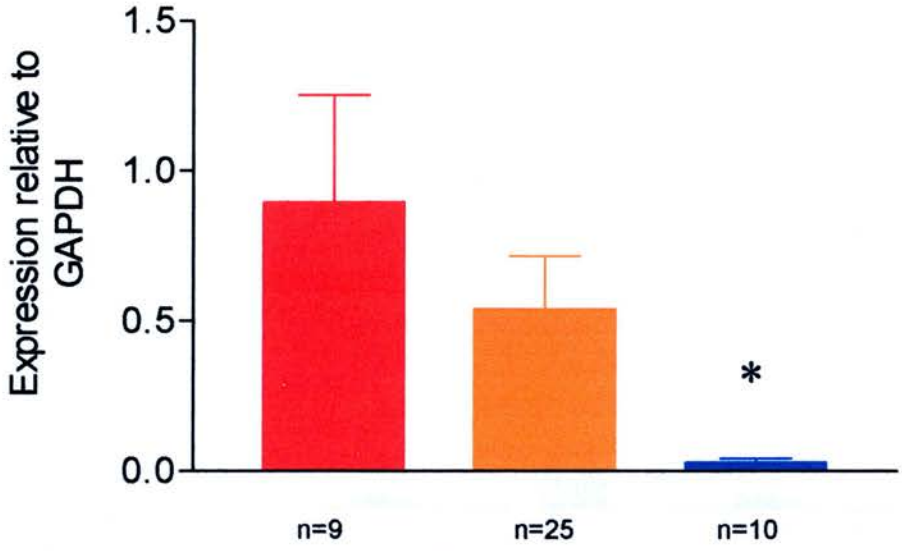
Expression of IL-4 was largely restricted to atopic skin. Significantly higher levels of IL-4 mRNA were detected in both lesional and non-lesional atopic skin compared to healthy skin ($p<0.05$) (Figure 7.4a), although there was no significant difference in expression between lesional and non-lesional atopic skin. IL-6 mRNA expression was also greatest in atopic skin (Figure 7.4b). However, IL-6 was also expressed in healthy skin and differences between the three groups failed to reach significance.

Significantly higher levels of IL-4 mRNA were detected in *D. farinae* stimulated atopic PBMCs compared to healthy PBMCs (Figure 7.5a). In contrast, much lower levels were detected in resting and ConA stimulated PBMCs, and no significant differences between atopic and healthy PBMCs were noted. IL-6 mRNA expression by PBMCs was highly variable between individuals (Figure 7.5b). Stimulation with ConA or *D. farinae* increased expression compared to resting cells, but levels in atopic and healthy PBMCs were otherwise very similar.

Figure 7.4 - Abundance of T_{H2} cytokine gene transcripts in atopic and healthy canine skin

■ - Lesional atopic dermatitis; ■ - non-lesional atopic dermatitis; ■ - healthy skin; bar - standard error of the mean; * - $p<0.05$.

a – IL-4



b – IL-6

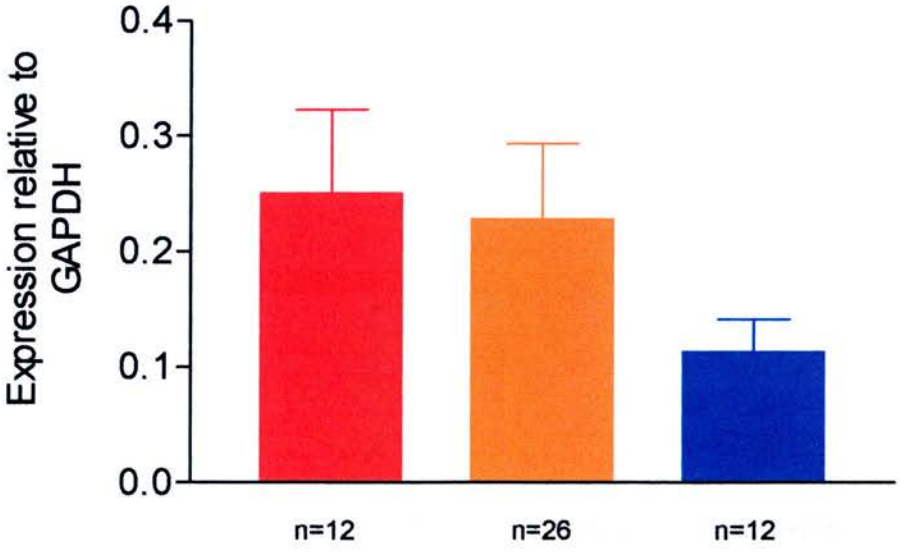
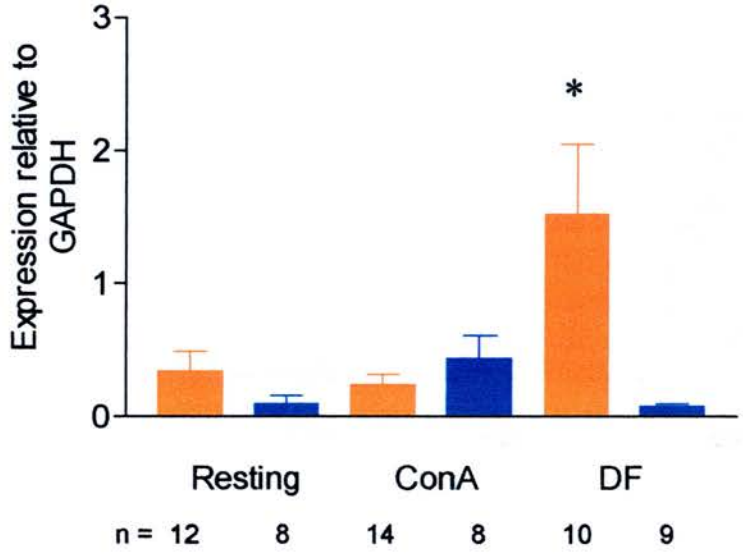


Figure 7.5 – Abundance of T_{H2} cytokine gene transcripts in atopic and healthy canine PBMCs

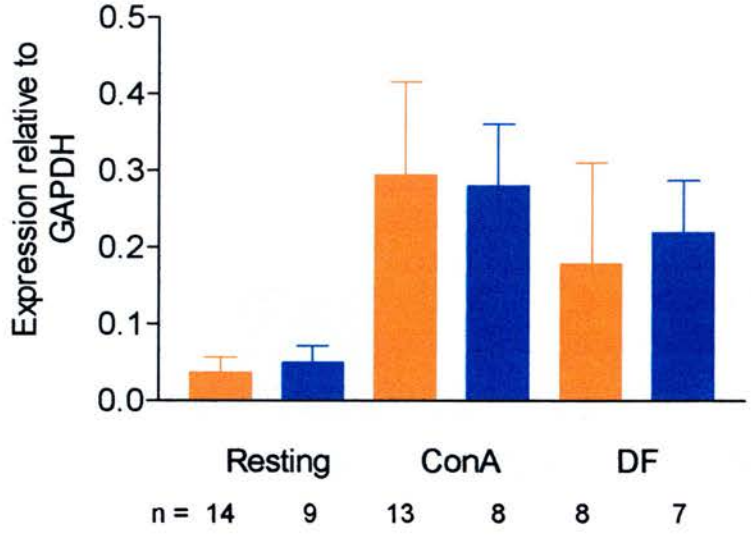
■ - Atopic PBMCs; ■ - Healthy PBMCs; bar - standard error of the mean;

* - $p<0.05$; resting – freshly isolated cells; ConA – cultured with 0.5μg/ml concanavalin A; DF – cultured with 50μg/ml Greer *D. farinae* extract.

a – IL-4



b – IL-6



7.3.3.2 Expression of T_{H1}-type cytokines

There was a similar pattern of mRNA expression for the T_{H1}-type cytokines IFNγ (Figure 7.6a), IL-2 (Figure 7.6b) and TNFα (Figure 7.6c) in all three groups of skin samples. Significantly higher levels of mRNA for each cytokine were detected in lesional atopic skin compared to either non-lesional atopic or healthy skin ($p<0.05$). Variably low levels, which did not significantly differ, were detected in non-lesional and healthy skin.

The pattern of transcription of these T_{H1} cytokines in PBMCs was more complex. There was a significantly greater level of IFNγ transcription in *D. farinae* stimulated healthy PBMCs compared to atopic PBMCs ($p<0.05$) (Figure 7.7a). Levels of mRNA were also higher in resting and ConA stimulated PBMCs from healthy dogs, although the differences between groups did not reach significance. Levels of mRNA for IL-2 were significantly higher in resting PBMCs isolated from healthy compared to atopic dogs ($p<0.05$) (Figure 7.7b). IL-2 mRNA levels were also higher in ConA and *D. farinae* stimulated healthy PBMCS, but transcription was highly variable and differences between atopic and healthy PBMCs did not reach significance. Transcription of TNFα was greatest in resting cells from healthy dogs, and in stimulated cells from atopic dogs (Figure 7.7c). However, the degree of transcription was highly variable and the differences did not reach significance.

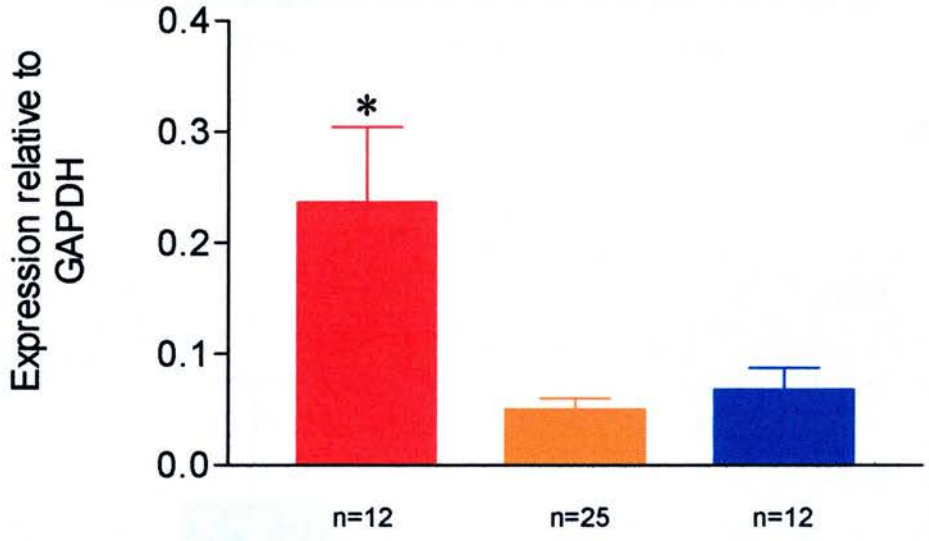
Levels of IL-12p35 and IL-12p40 mRNA were highly variable in both atopic and healthy skin. IL-12p35 mRNA expression did not appear to differ between lesional atopic, non-lesional atopic and healthy skin (Figure 7.6d). IL-12p40 mRNA expression was highest in lesional atopic and healthy skin, but there was no significant difference between the three groups of samples (Figure 7.6e).

There was no significant difference in IL-12p35 mRNA expression between resting PBMCs isolated from healthy and atopic dogs. In contrast, transcription was significantly higher in ConA and *D. farinae* stimulated cells from healthy dogs ($p<0.05$) (Figure 7.7d). However, IL-12p40 mRNA levels were highly variable between individuals, and no significant differences were observed between resting, ConA stimulated and *D. farinae* stimulated cells from atopic and healthy dogs (Figure 7.7e).

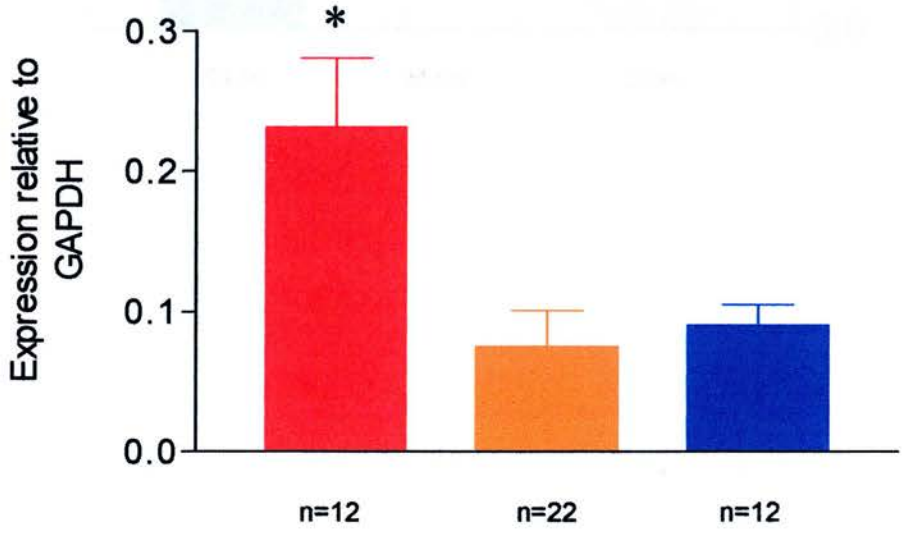
Figure 7.6 - Abundance of T_{H1} cytokine gene transcripts in atopic and healthy canine skin

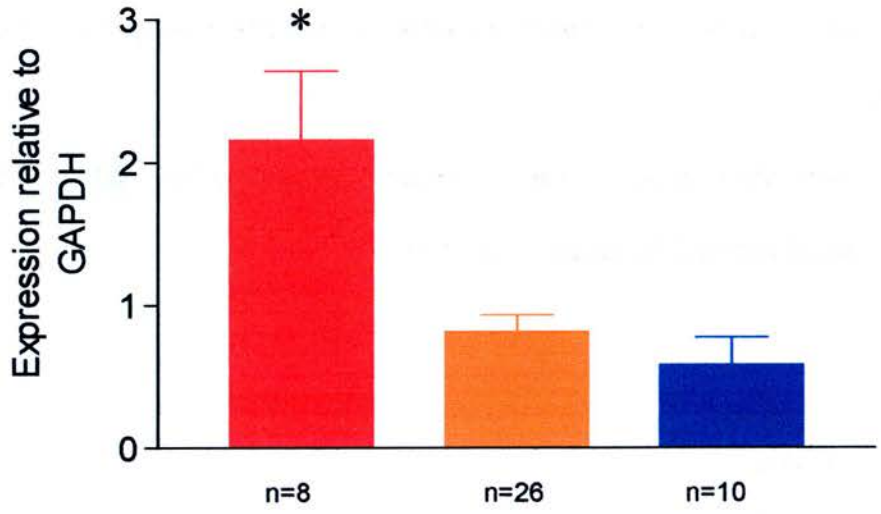
■ - Lesional atopic dermatitis; ■ - non-lesional atopic dermatitis; ■ - healthy skin; bar - standard error of the mean; * - $p<0.05$.

a – IFNγ

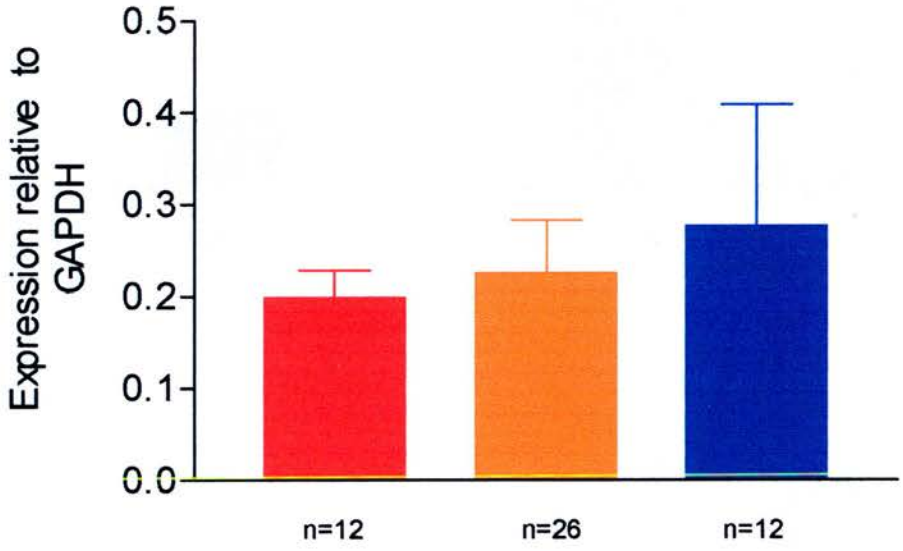


b – IL-2




Expression relative to GAPDH
3
2
1
0
*
n=8
n=26
n=10

d – IL-12p35


Expression relative to GAPDH
0.5
0.4
0.3
0.2
0.1
0.0
n=12
n=26
n=12

e – IL-12p40

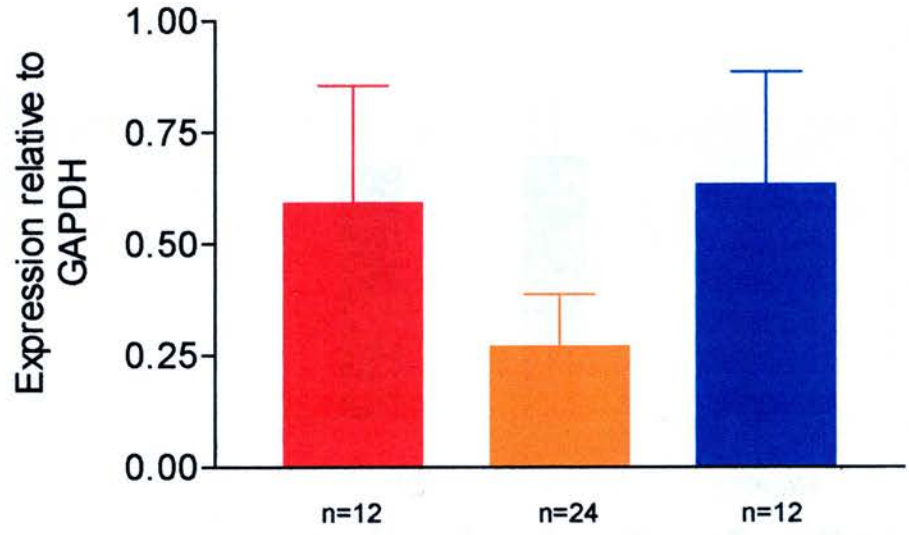
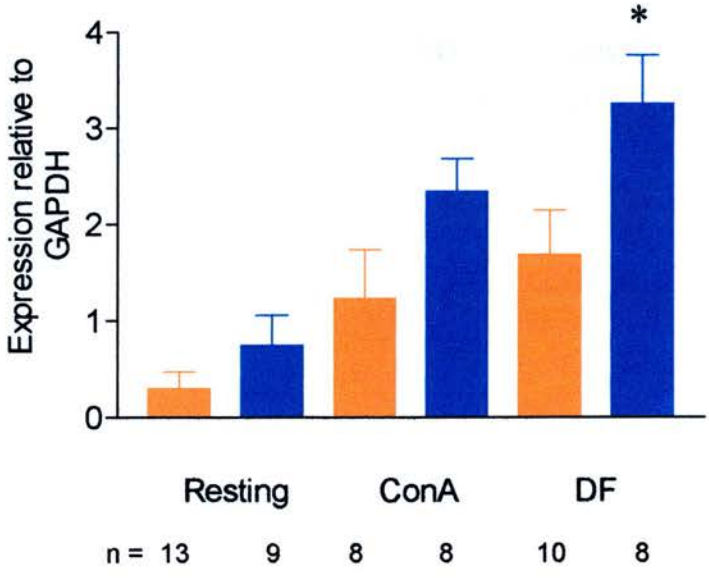


Figure 7.7 – Abundance of T_{H1} cytokine gene transcripts in atopic and healthy canine PBMCs

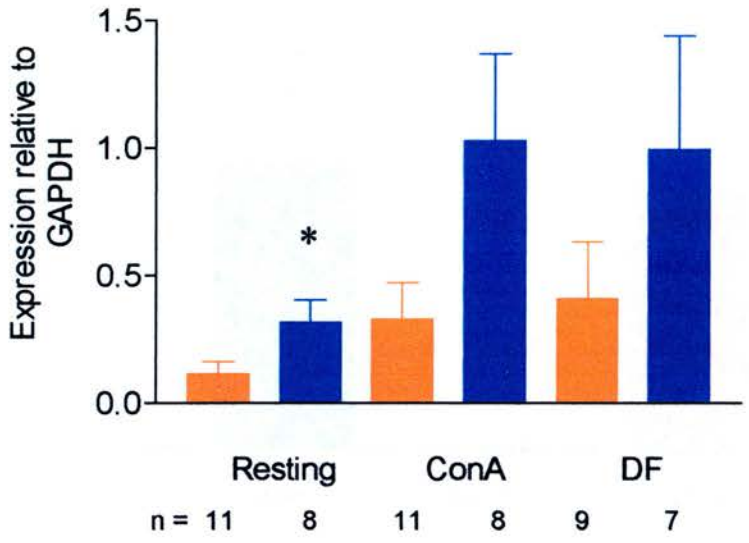
■ - Atopic PBMCs; ■ - Healthy PBMCs; bar - standard error of the mean;

* - $p<0.05$; resting – freshly isolated cells; ConA – cultured with 0.5μg/ml concanavalin A; DF – cultured with 50μg/ml Greer *D. farinae* extract.

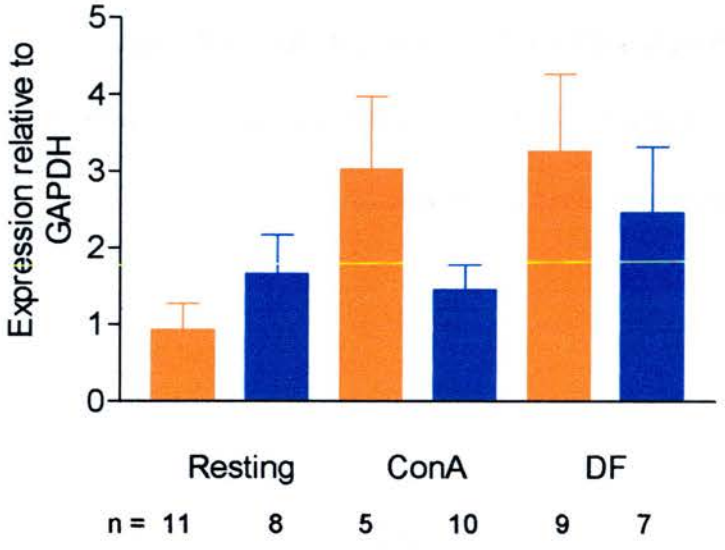
a – IFNγ



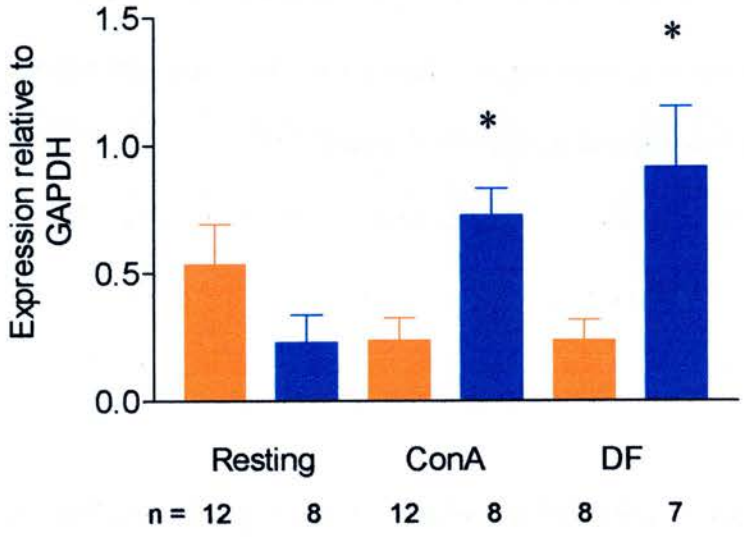
b – IL-2


Expression relative to GAPDH
1.5
1.0
0.5
0.0
*
Resting
ConA
DF
n = 11 8 11 8 9 7

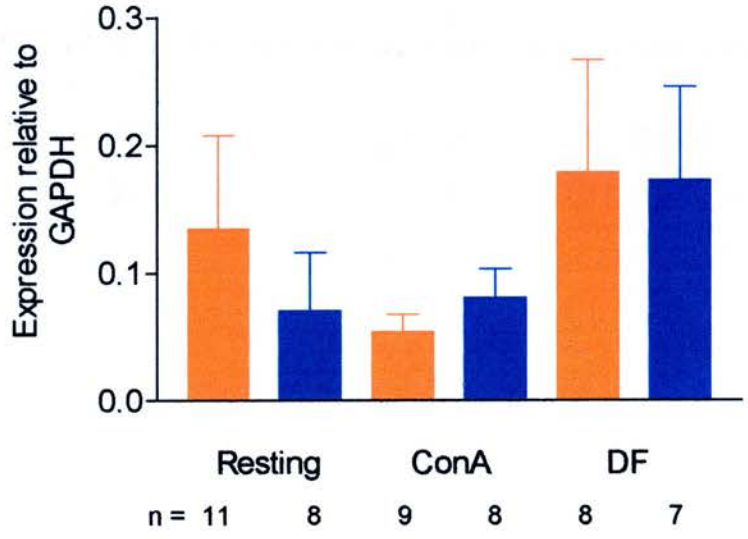
c – TNFα


Expression relative to GAPDH
5
4
3
2
1
0
Resting
ConA
DF
n = 11 8 5 10 9 7

d – IL-12p35



e – IL-12p40



7.3.3.3 *Immunosuppressive cytokines*

Healthy skin was associated with expression of the immunosuppressive cytokine TGFβ1. Levels of TGFβ1 mRNA were significantly higher in healthy compared with atopic skin (Figure 7.8a). There was, however, no significant difference between the lower levels of expression in lesional and non-lesional atopic skin.

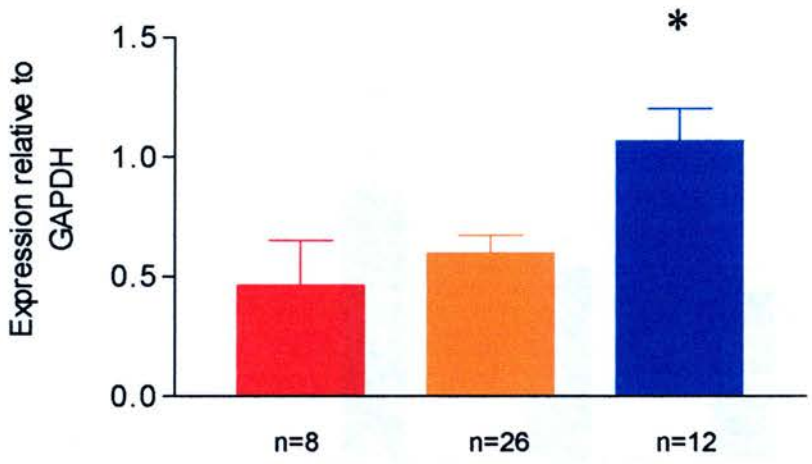
Levels of TGFβ1 mRNA were very similar in resting and ConA stimulated PBMCs isolated from atopic and healthy dogs (Figure 7.9a). Levels in *D. farinae* stimulated PBMCs, however, were significantly higher in healthy dogs compared to atopic dogs ($p<0.05$).

Levels of mRNA for IL-10 in skin were highly variable, and no significant difference in expression was detected between any of the three groups (Figure 7.8b). In contrast, IL-10 transcription was significantly higher in both ConA and *D. farinae* stimulated PBMCs isolated from healthy dogs compared to those from atopic dogs (Figure 7.9b) ($p<0.05$). Transcription in resting cells, however, was similar in healthy and atopic dogs.

Figure 7.8 - Abundance of immunosuppressive cytokine gene transcripts in atopic and healthy canine skin

■ - Lesional atopic dermatitis; ■ - non-lesional atopic dermatitis; ■ - healthy skin; bar - standard error of the mean; * - $p<0.05$.

a - TGFβ1



b – IL-10

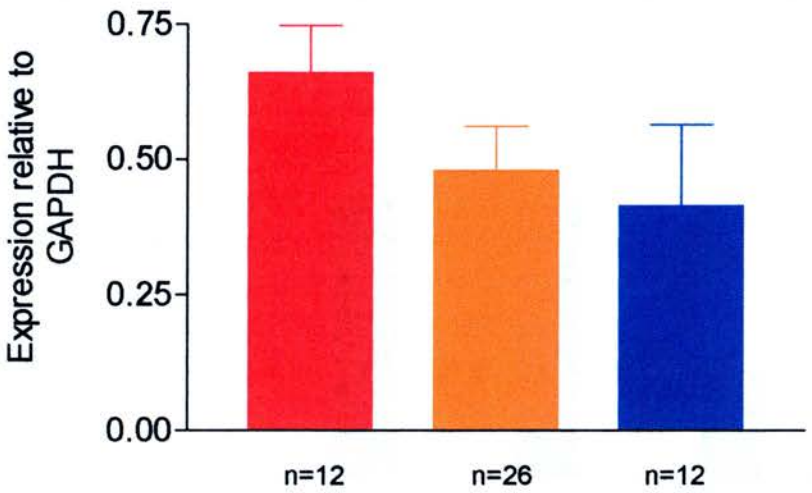
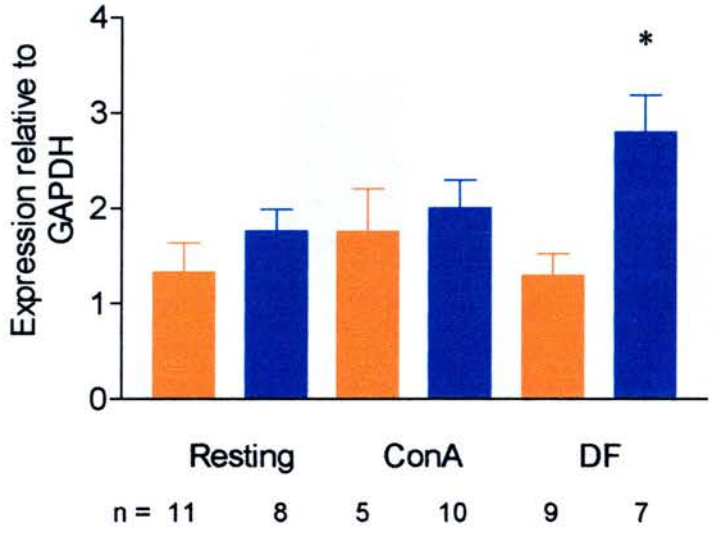


Figure 7.9 – Abundance of immunosuppressive cytokine gene transcripts in atopic and healthy canine PBMCs

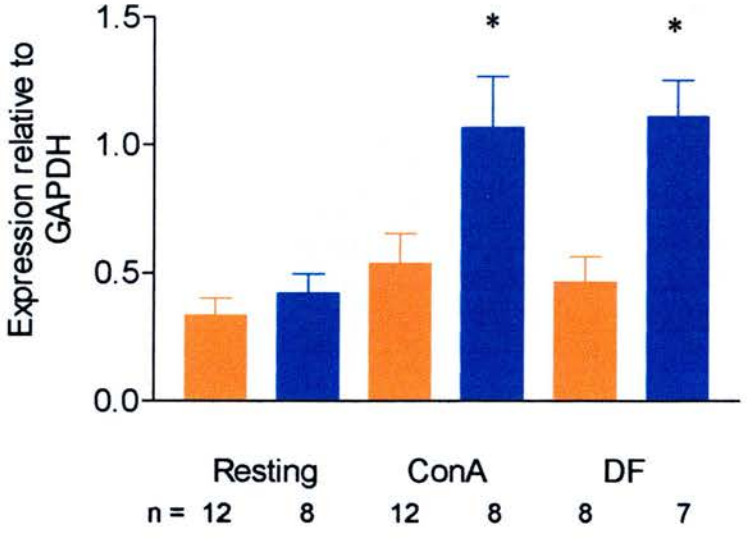
■ - Atopic PBMCs; ■ - Healthy PBMCs; bar - standard error of the mean;

* - $p<0.05$; resting – freshly isolated cells; ConA – cultured with 0.5μg/ml concanavalin A; DF – cultured with 50μg/ml Greer *D. farinae* extract.

a – TGFβ1



b – IL-10



7.3.3.4 *SCF and* c-Kit

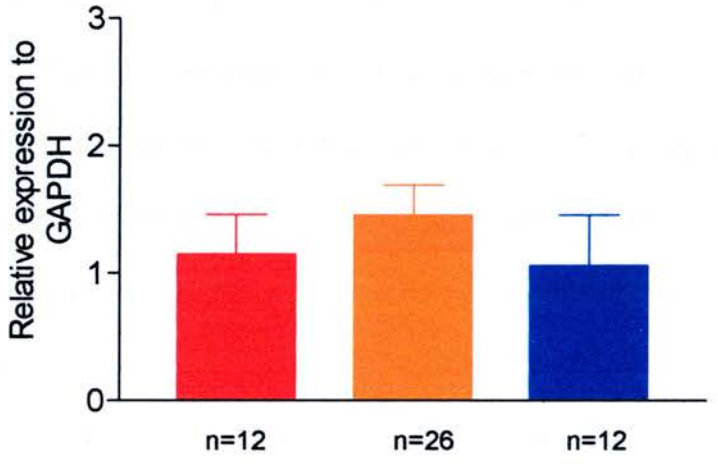
The level of SCF mRNA expression was very similar between all three groups of skin samples (Figure 7.10a). In contrast, gene transcription was consistently higher in resting, ConA and *D. farinae* stimulated PBMCs isolated from atopic dogs compared to those from healthy dogs (Figure 7.11a). The level of expression was very variable between individuals, however, and the differences were not statistically significant.

Expression of mRNA for *c-Kit* was highest in lesional atopic skin. Although the expression was quite variable between individuals, there was a significantly higher level of gene transcription in lesional atopic compared to healthy skin (Figure 7.10b). There were, however, no significant differences between non-lesional atopic skin and either lesional atopic or healthy skin. Levels of *c-Kit* mRNA expression in PBMC cultures were very variable between individuals. In contrast to the skin samples, significantly higher levels were observed in resting healthy compared to atopic cells. Despite this, levels in Con A and *D. farinae* stimulated atopic and healthy cells were comparable (Figure 7.11b).

Figure 7.10 - Abundance of SCF and *c-Kit* gene transcripts in atopic and healthy canine skin

■ - Lesional atopic dermatitis; ■ - non-lesional atopic dermatitis; ■ - healthy skin; bar - standard error of the mean; * - $p<0.05$.

a - SCF



b – *c-Kit*

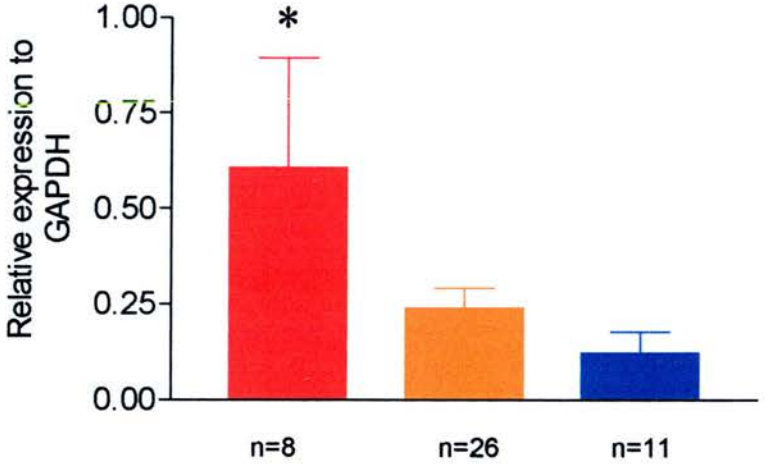
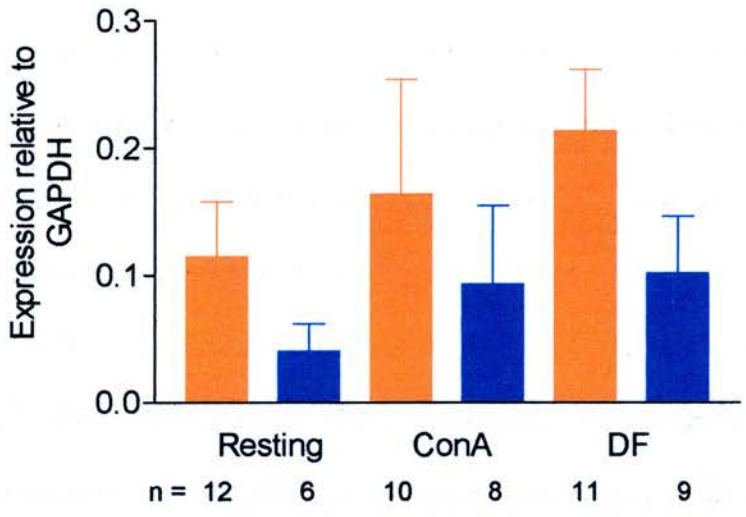


Figure 7.11 – Abundance of mast cell factor gene transcripts in atopic and healthy canine PBMCs

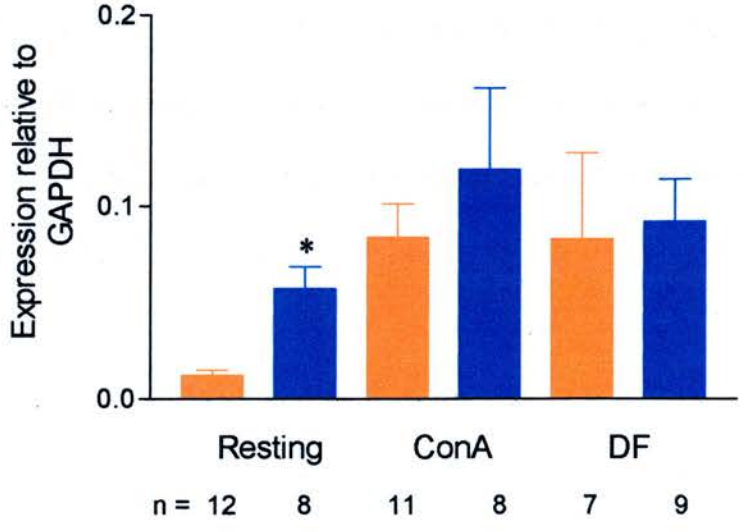
■ - Atopic PBMCs; ■ - Healthy PBMCs; bar - standard error of the mean;

* - $p<0.05$; resting – freshly isolated cells; ConA – cultured with 0.5µg/ml concanavalin A; DF – cultured with 50µg/ml Greer *D. farinae* extract.

a – SCF



b – *c-Kit*



7.4 Discussion

7.4.1 T_{H2} responses in canine atopic dermatitis

Studies in humans and experimental mouse models suggest that IL-4 expression is a hallmark of atopic dermatitis (Leung 2000). IL-4$^+$ CD4$^+$ T-cells are also found in feline allergic skin disease (Roosje and others 2002). In dogs, a non-quantitative study found that IL-4 mRNA was expressed in both atopic and healthy skin samples (Olivry and others 1999b). Using semi-quantitative RT-PCR, however, this study has shown that much higher levels of IL-4 mRNA are expressed in skin and PBMCs from dogs with atopic dermatitis compared to healthy dogs. These results suggest that IL-4 is likely to be involved in the pathogenesis of canine atopic dermatitis. This implies that human and canine atopic dermatitis share a similar pathogenesis and that the dog may be a naturally occurring model for human atopic dermatitis. In contrast to these findings, however, one study using quantitative RT-PCR failed to demonstrate IL-4 mRNA in canine skin (Maeda and others 2002). This dichotomy may represent differences in methodology, case selection or gene pools. Immunohistochemistry and proteomic analysis should, in the future, confirm whether there is expression of IL-4 protein in canine skin. In some dogs, however, T_{H2} polarisation and IgE production could be associated with IL-13. We were unable to identify IL-13 mRNA in control samples using consensus primers derived from human, murine, rat and bovine IL-13 sequences. A canine IL-13 sequence has now been published (Genbank® accession number AF244915 - www.ncbi.nlm.nih.gov) and further studies should reveal whether this cytokine has a role in canine atopic dermatitis.

This study found high levels of IL-4 mRNA expression in both non-lesional and lesional atopic skin. IL-4 is also found in non-lesional skin from human patients

(Leung 2000). It is not clear, however, if IL-4 expression in atopic skin is constitutive or as a result of exposure to specific allergens. Evidence of default T_{H2} responses comes from the impaired delayed type IV hypersensitivity responses observed in atopic humans and animals (Cooper 1994). Constitutive expression of IL-4 in mice, furthermore, is associated with increased numbers of mast cells, Langerhans' cells, acanthosis and hyperkeratosis (Elbe-Bürger and others 2002), which might enhance cutaneous sensitisation and inflammation. In this study, however, differences in the level of IL-4 mRNA expression in atopic and healthy PBMCs appeared to be allergen specific. Levels in resting and mitogen stimulated PBMCs, in contrast, were similar in both atopic and healthy dogs. These findings suggest that T_{H2} responses in atopic dogs are the result of interaction with specific allergens rather than the result of global T_{H2} polarisation. Studies in humans have shown that T_{H1} and T_{H2} responses co-exist in the same individual, further suggesting that these responses are antigen specific (Ismail and Bretscher 1999). In addition, T_{H2} responses in atopic patients are allergen specific and are not associated with non-sensitising antigens (Jenmalm and others 2001). Certain allergens may drive T_{H2} differentiation. Der p 1, for example, increases epithelial permeability, enhances protease activity, IgE production, inflammatory mediator release and inhibits T_{H1} differentiation (Bufe 1998; Shakib and Gough 2000). Studies on the *in vivo* biological activity of group 1 allergens and Der f 15 on T_{H2} differentiation in atopic dogs are therefore warranted.

Allergen specific IgE and PBMC responses are largely restricted to sensitised atopic dogs (Nuttall and others 2001a; Nuttall and others 2001b). The findings from this study suggest that following allergen exposure these cells express IL-4, which drives allergen specific IgE production. It is not, however, clear from these results which

cells are expressing IL-4 mRNA. Further studies to phenotype the allergen reactive and IL-4 mRNA and protein expressing cells are therefore required.

In some atopic dogs intradermal test reactivity can be demonstrated in the absence of detectable circulating IgE. IL-4 expression in atopic canine skin suggests that IgE production might take place in the skin. However, the paucity of B-cells and plasma cells in the cutaneous mononuclear cell infiltrate in canine atopic dermatitis (Olivry and others 1997), suggests that most IgE production takes place at extra-cutaneous lymphoid sites (Hill and Olivry 2001). Immunohistochemistry, furthermore, indicates that most IL-4 producing cells in the dermis are mast cells, rather than T-helper cells (Sinke and others 2002).

Consistent with the hypothesis that IL-4 expression is associated with IgE production, elevated IL-4 levels are not found in the intrinsic (non-IgE mediated) form of human atopic dermatitis (Schmid-Grendelmeier and others 2001). The findings described in Chapters 3 and 6 suggest that IgE and non-IgE mediated forms of canine atopic dermatitis also exist. Unfortunately, it was not possible to evaluate gene transcription in samples from non-allergen reactive dogs in this study. Further studies are therefore needed to definitively link IL-4 expression, IgE production and allergen reactivity in canine atopic dermatitis.

In contrast to a previous investigation (Olivry and others 1999b), IL-6 mRNA was readily detected in this study. However, unlike IL-4, there was no significant difference in expression between atopic and healthy canine skin, resting and mitogen or allergen stimulated PBMCs. Although IL-6 is regarded as a T_{H2}-type cytokine (Maggi 1998), it is also produced by APCs, fibroblasts, macrophages, endothelial cells and keratinocytes (Kirman and Le Gros 1998). In a mouse model IL-6 was

shown to have both pro- and anti-inflammatory effects (Wang and others 2000b). The activity of IL-6 may, therefore, depend on the precise temporal and spatial relationship between cells. Furthermore, IL-6 could support B-cell proliferation and allergen specific IgG production in healthy humans and dogs (Upham and others 1995; Day and others 1996).

7.4.2 Immunosuppressive cytokines in canine atopic dermatitis

This study has shown for the first time that there are lower levels of gene transcription for the immunosuppressive cytokines TGFβ1 and IL-10 in atopic compared to healthy dogs. This could result in the altered skin reactivity that has been associated with clinical atopic dermatitis (Hyland 2001; Sinke and others 2002). A recent study, furthermore, linked a low TGFβ1 producer phenotype with atopic dermatitis in children (Arkwright and others 2001).

TGFβ1, produced by T-cells and keratinocytes, is thought to be important in peripheral tolerance to environmental allergens in humans and rodent models (Yeo and Lamb 1995). Atopic dermatitis in humans and dogs is associated with hyperreactivity to irritant stimuli (Leung 2000; Scott and others 2001a). The low level of cutaneous TGFβ1 mRNA found in this study could, therefore, reflect a breakdown of anti-inflammatory mechanisms. In contrast, constitutive expression of TGFβ1 in healthy skin could inhibit inappropriate inflammatory responses to environmental allergens. In a murine model of atopic dermatitis, administration of recombinant $TGF\beta_1$ resulted in decreased serum IgE levels, IFNγ production and amelioration of skin lesions although it also caused acanthosis and fibrosis (Sumiyoshi and others 2002). The balance of its anti-inflammatory and stimulatory activity may, therefore,

depend on the degree of differentiation or activation of a target cell as well as the overall cytokine and cellular milieu (Ling and Robinson 2002). TGFβ1, however, is secreted in an inactive form and is subject to post-transcription regulation (Umetsu and DeKruyff 1997; Ling and Robinson 2002). Confirmation of its importance, therefore, awaits demonstration of active TGFβ1 in canine skin and PBMCs.

The role of IL-10 in canine skin is less clear. An earlier investigation failed to demonstrate IL-10 mRNA in canine skin (Olivry and others 1999b). In contrast, IL-10 mRNA was expressed in most samples in this study, although there was no difference in the level of expression between atopic and healthy skin. The role of IL-10 is also unclear in humans and rodent models. Decreased IL-10 expression has been associated with colitis and cutaneous inflammation in mice (Borish 1998), and atopic dermatitis in people (Koning and others 1997b; Jenmalm and others 2001). In contrast, increased IL-10 expression has been found in T_H cells from patients with atopic dermatitis (Sato and others 1998) and in the airways and skin after allergen challenge in mice and humans (Koulis and Robinson 2000; van Scott and others 2000). IL-10 is constitutively expressed in normal mouse skin, but only in late phase reactions in a model of atopic dermatitis (Wang and others 1999), which may fail to resolve established allergen induced inflammation (Borish 1998; Muraille and Leo 1998).

Significantly greater TGFβ1 and IL-10 gene expression was observed in allergen stimulated PBMCs from healthy compared to atopic dogs. TGFβ1 and IL-10 inhibit the proliferation and activation of T-cells (Herz and others 1998), which may account for the anergic response in PMBCs from healthy dogs to *Dermatophagoides* allergens (Nuttall and others 2001b). An early study also reported that PBMCs isolated from atopic dogs exhibited less suppressor activity than those from healthy dogs

(Nimmo Wilkie and others 1992). Allergen induced IL-10 and TGFβ1 expression in canine PBMCs is consistent with activation of T-regulatory cells in healthy individuals. It has been proposed that these play an important role in tolerance to environmental allergens (Hoyne and others 2000). Low IL-10 mRNA expression in the mitogen stimulated PBMCs from atopic dogs, furthermore, might indicate there is a constitutive defect in immunosuppressive gene expression in these individuals. This was not, however, associated with widespread dysregulation, as IL-10 mRNA expression was readily detected in atopic skin. Low expression in PBMCs, however, may allow unregulated activation of T_H cells and a breakdown in tolerance.

7.4.3 The role of T_{H1} cytokines in canine atopic dermatitis

High levels of mRNA for the T_{H1} cytokines IL-2, IFNγ and TNFα were seen in lesional atopic skin. Chronic lesions in human atopic dermatitis also exhibit a mixed pattern of cytokines, including IL-4, IL-13, IL-5, IL-2, IFNγ, and IL-12 (Werfel and others 1996). Furthermore, T_{H1} cytokines dominate atopy patch tests after 48 hours (Thepen and others 1996; Junghans and others 1998). Another recent study has also shown that IFNγ and TNFα, as well as IL-1β and TARC (Thymus and Activation Regulated Chemokine), are up regulated in lesional atopic compared to non-lesional atopic and healthy canine skin (Maeda and others 2002).

T_{H1} cytokines appear, therefore, to be important in the establishment and maintenance of chronic lesions. IL-2 is a crucial factor in the activation and expansion of T-cells (Huston 1997). Lesional skin in humans is characterised by Fas-dependant keratinocyte apoptosis and disruption of the epidermal barrier, increasing exposure to allergens, irritants and microbes. IFNγ, in particular, induces expression of Fas on

keratinocytes, which interacts with Fas-ligand present on infiltrating T-cells (Trautmann and others 2000). In one study, keratinocytes from humans with atopic dermatitis were more sensitive to IFNγ than those from healthy individuals (Pastore and others 1998). TNFα produced by human and rodent epidermal cells triggers expression of adhesion molecules including ICAM-1 and VCAM-1 as well as pro-inflammatory cytokines and chemokines (Robert and Kupper 1999). TNFα also stimulates Langerhans cell activation, maturation, migration, antigen presentation and survival (Kimber and others 1998).

The trigger for T_{H1} cytokine expression in chronic lesions is not fully understood. Skin lesions in human and canine atopic dermatitis include erythema, papular eruptions, alopecia, scaling, crusting, hyperpigmentation and lichenification (Rothe and Grant-Kels 1996; Scott and others 2001a). However, the clinical findings described in Chapter 3 suggest that uncomplicated canine atopic dermatitis is largely a pruritic and erythematous disease. Other authors have also concluded that most lesions result from self-trauma and microbial colonisation (Rothe and Grant-Kels 1996; Griffin and DeBoer 2001). Self-trauma can induce inflammatory cytokines, including IL-1, TNFα and GM-CSF from keratinocytes (Robert and Kupper 1999) that could result in the recruitment of mononuclear cells and the development of chronic lesions. Histamine, furthermore, can promote T_{H1} proliferation and inhibit T_{H2} cells *in vitro* (Jutel and others 2001).

These results clearly demonstrate that T_{H1} cytokine mRNA is up regulated in lesional skin. It is tempting to surmise that there is a correlation between the severity of inflammation and T_{H1} cytokine expression. Further study comparing the degree of cel-

lular infiltration into the skin with the level of T_{H1} cytokine mRNA expression will begin to answer this question.

It was suggested in Chapter 3 that lesional atopic dermatitis is associated with secondary infection. The severity of human atopic dermatitis is also associated with staphylococcal colonisation (Herz and others 1998). In a mouse model, furthermore, *Staphylococcus aureus* preferentially bound to sites of IL-4 mediated inflammation (Cho and others 2001), suggesting that they play an important role in the switch from T_{H2} to T_{H1}-type inflammatory reactions. Staphylococcal superantigens in humans induce CLA expression on T-cells, MHCII, IL-1, TNFα and IL-12 expression by mononuclear cells and up-regulate endothelial ICAM-1 and VCAM-1 (Herz and others 1998; Leung 2000; Skov and others 2000). The role of staphylococcal superantigens in canine atopic dermatitis, however, has not yet been studied.

Superantigen activity in *Malassezia* species has not been demonstrated, but *Malassezia* have been shown to induce IgG, IgE, immediate and late phase skin reactivity and PBMC proliferation in human (Kieffer and others 1990; Nordvall and Johansson 1990; Rokugo and others 1990; Wessels and others 1991; Tengvall Linder and others 1998) and canine (Morris and others 1998; Nuttall and Halliwell 2001; Chen and others 2002) atopic dermatitis. *Malassezia* also induce IL-1β, IL-6, IL-8 and TNFα production from human keratinocytes (Watanabe and others 2001). *M. pachydermatis* appeared to be more pathogenic than *M. furfur* in this study, but this may reflect host adaptation, rather than virulence.

Together, these findings suggest an important role for micro-organisms in enhancing leucocyte adhesion and migration, pro-inflammatory cytokine release and the development of chronic skin lesions.

The T_{H1}-type cytokines IL-2, IFNγ and TNFα are associated with cell-mediated inflammation and inhibition of T_{H2} differentiation (Fiorentino and others 1989). These results, however, do not suggest that T_{H2} skewing in canine atopic dermatitis results from defective expression of T_{H1} cytokines. There was, however, significantly greater transcription of IFNγ by allergen stimulated PBMCs from healthy dogs, suggesting that there is a T_{H2} polarised circulating allergen specific PBMC population in atopic dogs. A recent study also found decreased IFNγ production in PBMCs isolated from IgE associated but not non-IgE associated human atopic dermatitis (Simon and others 2002). Compared to healthy children, furthermore, atopic children not only have fewer IFNγ producing $CD4^+$, $CD8^+$ and NK cells in their peripheral blood, but they also expressed less IFNγ per cell (Campbell and others 1999).

APC derived IL-12 is a critical factor in T_{H1} differentiation, IFNγ production, and CLA expression on circulating T-cells (Blaher and others 1995; De Becker and others 1998; Snijders and others 1998). Down regulation of IL-12 has been associated with T_{H2} polarisation (Muraille and Leo 1998), but there was no significant difference in the overall expression of IL-12 mRNA in healthy and atopic canine skin in this study. Whilst this does not preclude post-transcription down regulation of IL-12 production or function, T_{H1}-type cytokine expression in lesional skin suggests that this is not the case. IL-12p35 transcription was, however, significantly lower in mitogen and allergen stimulated PBMCs from atopic compared to healthy dogs. Reduced IL-12p35 expression in atopic PBMCs could favour T_{H2} differentiation as IL-12p40 dimers inhibit IL-12 activity (Piccotti and others 1996).

The findings in this study do not suggest that expression of T_{H1} cytokines down regulates T_{H2} expression in healthy individuals. An earlier non-quantitative investigation

detected IL-2 expression more frequently in healthy than atopic canine skin samples, although the numbers expressing IFNγ and TNFα did not differ (Olivry and others 1999b). In contrast, only low levels of IL-2, IFNγ and TNFα mRNA were expressed in healthy canine skin in this study. These results are not consistent with T_{H1} polarisation in healthy skin, although it would unusual to find high levels of these pro-inflammatory cytokines expressed in healthy skin. This further supports the hypothesis that it is expression of the immunosuppressive cytokines IL-10 and TGFβ1 that is important in tolerance.

7.4.4 The expression of SCF and *c-Kit* in canine atopic dermatitis

In human atopic dermatitis there is a correlation between SCF expression, mast cell numbers and dermal histamine content (Rossi and others 1998; Galli and others 1999). An immunohistochemical study also found higher levels of extracellular SCF protein in atopic compared to healthy canine skin (Hammerberg and others 2001). Labelling was particularly intense in lesional skin. Despite this, there was little difference in the expression of SCF mRNA between atopic and healthy dogs in this study. Immunohistochemistry, however, is more sensitive than RT-PCR at detecting preformed, stored and released mediators.

As well supporting mast cell development, SCF potentiates IgE dependent degranulation in rat mast cells (Hill and others 1996) and both IgE dependent and independent mast cell activation in atopic humans (Brody and Metcalfe 1998; Galli and others 1999; Kanbe and others 1999). However, whilst one study has shown that SCF augments mast cell activation in dogs (Brazís and others 2000), in another it did not (Hammerberg and others 2001). The latter demonstrated that anti-IgE increased in-

tradermal test wheal diameter in atopic but not healthy dogs, although earlier work found that *in vitro* sensitised mast cells from atopic and normal canine skin had a similar response to anti-IgE (Brazís and others 1998). This suggests that *in vivo* sensitisation was more important than inherent activity. Mast cell involvement in atopic dermatitis, therefore, may be secondary to allergen specific IgE production driven by T_{H2} differentiation.

The study also found increased *c-Kit* mRNA transcription in lesional atopic skin. Expression of *c-Kit* mRNA was, conversely, decreased in atopic compared to healthy PBMCs. This may be because gene transcription in PBMCs was very low and image analysis less accurate. Increased cutaneous *c-Kit* mRNA expression may be due to mast cell hyperplasia, although other work suggests that mast cell numbers are not significantly elevated in atopic compared to healthy skin (Hammerberg and others 2001). Increased *c-Kit* expression could also be a pathway to enhance SCF activity in lesional atopic skin.

7.4.5 The pathogenesis of canine atopic dermatitis

These results allow us to postulate a pathogenetic sequence for canine atopic dermatitis. Activation of T_{H2} polarised cells promotes allergen specific IgE production in atopic dogs, whereas IL-10 and TGFβ1 expression induces tolerance in healthy individuals. Atopic skin, furthermore, is associated with increased expression of IL-4 and decreased expression of TGFβ1, leading to allergic-type inflammatory reactions. With chronicity, lesional skin exhibits a mixed inflammatory cytokine pattern that may be due to self-trauma and secondary infection. Ongoing exposure to environmental allergens sets in train a vicious circle of inflammation, trauma and microbial

infection that fails to resolve. Some caution should be exercised, however, as semi-quantitative RT-PCR is only an approximate measure of mRNA expression. Techniques that accurately quantify mRNA are more accurate and should be applied to confirm some of these findings.

8. Conclusions

8.1 Introduction

This thesis describes the clinical and immunological features of 45 dogs with confirmed atopic dermatitis. Until very recently little was known of the pathogenesis of this disease, although the presence of circulating and cutaneous mast cell bound allergen specific IgE was well recognised. The most common allergens are the house dust mites *Dermatophagoides farinae* and *D. pteronyssinus*, but the major protein allergen for atopic dogs seems to be a 98kD chitinase (Der f 15) rather than group 1 or 2 allergens (Noli and others 1996; McCall and others 2000; Shaw 2000). It is also becoming apparent that canine atopic dermatitis is a T-cell mediated disease. $CD4^+$ and $CD8^+$ T-cells dominate the cutaneous infiltrate (Olivry and others 1997) and *D. pteronyssinus* specific PBMC proliferation was demonstrated *in vitro* (Shaw 2000). Analysis of the cytokine profile and T_{H1}/T_{H2} polarisation in canine atopic dermatitis has been limited though. One study using RT-PCR demonstrated a T_{H1}-like pattern in 25% of healthy skin samples, a T_{H2}-like pattern in 25% of atopic skin samples and a mixed cytokine pattern in the rest (Olivry and others 1999b). Others detected IL-4 in atopic lesional skin by immunohistochemistry (Sinke and others 1998) and patch test sites (Rutten and others 2001). Despite this, a recent study failed to detect IL-4 (Maeda and others 2002) in atopic skin. Other authors speculated that T_{H1}-type cell mediated inflammatory reactions also participated in the pathogenesis of chronic atopic dermatitis (Olivry and others 1999b; Sinke and others 2002).

The findings in this thesis have extended our knowledge in these key areas. Canine atopic dermatitis appeared be an allergen mediated disease in most of the studied dogs. It was strongly associated with intradermal test reactivity, allergen specific se-

rum IgE and *in vitro* PBMC proliferation to the house dust mites *D. farinae* and *D. pteronyssinus* (Nuttall and others 2001a; Nuttall and others 2001b). The immunoblotting and PBMC proliferation studies, furthermore, demonstrated that the major target of immune recognition in atopic dogs is a 98/104kD allergen that is probably Der f 15 (Nuttall and others 2001a; Nuttall and others 2002a). The correlation between the presence of allergen specific IgE and PBMC proliferation implies that these cells support IgE production, confirming the role of T-cells in the pathogenesis of atopic dermatitis. Semi-quantitative RT-PCR specific for canine cytokines was used to investigate cytokine profiles in skin and PBMCs. This demonstrated increased expression of the mRNA encoding the T_{H2} cytokine IL-4 and decreased expression of the mRNA encoding the immunosuppressive cytokine TGFβ1 in atopic skin. Expression of mRNA for the T_{H1} cytokines IFNγ, TNFα and IL-2 was restricted to lesional atopic skin (Nuttall and others 2002b). *In vitro* culture of atopic PBMCs with an extract of *D. farinae* induced up regulation of IL-4 mRNA and down regulation of mRNA encoding the T_{H1} cytokine IFNγ. In contrast, allergen stimulation of healthy PBMCs induced expression of mRNA encoding the immunosuppressive cytokines TGFβ1 and IL-10.

8.2 The pathogenesis of canine atopic dermatitis

The findings suggest that canine atopic dermatitis is associated with a T_{H2} polarised phenotype that leads to the production of IgE antibodies specific to environmental allergens. Cross-linking of IgE antibodies on the surface of cutaneous mast cells triggers an immediate phase inflammatory reaction. The subsequent inflammatory cascade results in recruitment and activation of T_{H1}-type cells and chronic cell mediated

inflammation. Other factors that may be important in the development of chronic lesions include self-trauma and colonisation by staphylococcal and *Malassezia* species. Having both an atopic phenotype and a failure of peripheral tolerance, however, appear key to clinically manifesting the condition. Consistent with this hypothesis a recent study in high IgE-responder strain of beagles discovered that increased allergen specific serum IgE levels and allergen exposure were not sufficient by themselves to induce clinical signs of atopic dermatitis (Egli and others 2002). A breakdown in peripheral tolerance without an atopic constitution, in contrast, could explain why some dogs fulfil the clinical requirements for a diagnosis of atopic dermatitis, yet have persistently negative IDTs, serology and PBMC proliferation assays.

This hypothetical two step model of atopic dermatitis (i.e. atopic phenotype and a breakdown of peripheral tolerance) could be tested by studying IDT positive healthy animals, which should express high levels of IL-4 and IL-10/TGFβ1, and IDT negative atopic dogs, which should have a low IL-4 and low IL-10/TGFβ1 phenotype.

8.3 Tolerance in healthy individuals

Human and murine studies have shown that TGFβ and IL-10 are critical in the induction of $CD4^+$ T-regulatory (T_R or T_{REG}) cells. Recent findings in a mouse model of peptide immunotherapy for Der p 1 induced responses revealed that TCR ligation in conjunction with IL-10 expression and activation of the Notch signalling pathway allows naïve T-cells to differentiate into T_R cells. These cells mediate tolerance by preventing the activation and expansion of T-cell clones. Furthermore, expression of Notch receptors and ligands is modulated on T-cells following exposure to immunosuppressive cytokines (Hoyne and others 2000). Investigating the role of Notch sig-

nalling in the regulation of immune responses in atopic and healthy dogs could, therefore, have major implications for our understanding of tolerance. Analysis of the relevant balance of immunosuppressive, T_{H2} or T_{H1} influences within an individual would be greatly enhanced by developing quantitative RT-PCR techniques to accurately determine the level of cytokine transcription.

Cytokine mRNA, however, does not necessarily correlate with expression of active protein as post-transcriptional regulation of expression and function can occur. Analysis of protein expression is hampered by the lack of canine specific reagents and the limiting nature of selecting likely targets *a priori*. An alternative approach is to use proteomics to study global protein expression in tissues from atopic and healthy dogs. Proteins with differential expression could then be sequenced, identified and studied further.

8.4 The role of *Dermatophagoides* allergens

The most prominent allergen in canine atopic dermatitis appears to be *D. farinae* (Sture and others 1995; Nuttall and others 2001a). This species, however, is believed to be rare in the UK. Cross inhibition studies will be required to determine whether antibodies to *D. pteronyssinus* allergens cross react with those from *D. farinae*. If there appears to be independent sensitisation to both species it raises the intriguing possibility that there may be an association between dogs and *D. farinae*. It would be interesting, therefore, to compare the prevalence of *D. farinae* and *D. pteronyssinus* as well as Der f 1 and Der p 1 in homes with and without dogs in different geographical areas of the UK. Alternatively, significant cross-reaction between these

two species has implications for allergen standardisation, as allergen tests will exaggerate the importance of extracts with high levels of reactive proteins.

The major allergen Der f 15 and its *D. pteronyssinus* homologue could be useful markers to standardise allergen extracts for use in atopic dogs. Investigating the effect purified Der f 15 has on canine mononuclear cell lines, PBMCs and skin explant cultures will also establish if there is any biological activity that could be important in the pathogenesis of canine atopic dermatitis. Group 1 allergens also warrant study as some dogs did react to these proteins. Der p 1 can also disrupt epithelial barriers, and promote T_{H2} polarisation and sensitisation to other allergens.

8.5 The role of microorganisms in canine atopic dermatitis

The triggers for the switch from T_{H2} to T_{H1} mediated inflammation are unclear. Clinically, however, lesional skin in atopic dogs was associated with secondary infection by staphylococci and *Malassezia*. This implies that these organisms are important in driving T_{H1} mediated chronic inflammation. They are commensal organisms, but although colonisation of atopic skin is common, no virulence factors have been found to distinguish isolates from infected and non-infected individuals (DeBoer and Marsella 2001). Traditional approaches, however, tend to focus on a few molecules. Using proteomic techniques it would be possible to screen microbial isolates for differences in protein expression. The identity and role of any potential virulence factors could then be studied in greater detail. If the isolates prove to be similar, though, this would imply that the cutaneous microenvironment is more important in microbial colonisation.

Once established, it is clear that microorganisms induce an inflammatory response. This could result from metabolic by-products and proinflammatory mediators. In addition, *Malassezia* and *Staphylococcus* specific IgG and IgE antibodies suggest that these organisms might act as conventional allergens in atopic dogs (Morales and others 1994; Morris and others 1998; Nuttall and Halliwell 2001; Chen and others 2002). Sensitisation could have major implications, as antimicrobial therapy might not be able to reduce the commensal populations below a critical threshold for sensitisation. The role of microbial superantigens in canine atopic dermatitis should also be explored. Staphylococcal superantigens and *Malassezia* extracts are potent inducers of PBMC proliferation in dogs (Hendricks and others 2002; Morris and others 2002), but the TCR Vβ chain profile of these cells has not been investigated.

8.6 The epidemiology of canine atopic dermatitis

The 45 dogs with atopic dermatitis in this study were drawn from 22 different breeds. Whilst this is far from a statistically valid sample, it does suggest that atopic dermatitis is a widespread condition. Several breeds are said to be at risk from the disease (Scott and others 2001a), but there is little robust epidemiological data to support this assertion. Individual dog breeds represent outbred and isolated gene pools. Studying the epidemiology of canine atopic dermatitis could, therefore, be a powerful tool to investigate the relative importance of different atopic traits between gene pools. Atopic traits in dogs remain poorly characterised although this study has identified a number of potential targets. Further studies are currently in progress to detect polymorphisms in DNA isolated from atopic and healthy dogs that might be associated with altered function in pro-inflammatory or immunosuppressive cytokines. These

studies will also show if specific MHCII haplotypes are associated with atopic dermatitis in dogs.

8.7 Conclusion

The findings reported here significantly advance our understanding of atopic dermatitis in dogs. Canine atopic dermatitis, furthermore, should be developed as a valuable, spontaneous model for studying the human disease. Establishing the immunological mechanisms underlying tolerance will allow canine atopic dermatitis to be further developed as a powerful model to explore new approaches to therapy in dogs and humans. Identifying key mediators will also allow the utilisation of molecular techniques to investigate genetic polymorphisms in between atopic and healthy dogs.

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

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SUMMARY

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

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

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

The identification of HDM allergens in canine AD is controversial. Studies using anti-canine IgGd (Noli et al 1996) and recombinant FcεRIα (McCall et al 2000, Shaw 2000) detected major allergens of 60–70 kDa and 90–109 kDa on Western blots of *D farinae* and *D pteronyssinus*. However, although 69 per cent of atopic sera also recognised Der f1 using FcεRIα in one study (McCall et al 1999), others detected very little binding to group 1 and 2 allergens using either FcεRIα (Shaw 2000) or anti-canine IgGd (Noli et al 1996). In contrast, the only study using anti-canine IgE (in ELISAs and dot blots) reported that 50 per cent of atopic sera recognised both Der p1 and 2, and Der f1 and 2 (Masuda et al 1999).

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

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

MATERIALS AND METHODS

Study population

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

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

All dogs with a clinical diagnosis of AD were skin-tested with 57 allergens, including *D farinae* and *D pteronyssinus* (Greer Laboratories, Lenoir, USA). Dogs were sedated with 0·15 mg kg^{-1} xylazine IM. Hair was clipped from the lateral flank and 0.05 ml of each allergen extract, 1/100,000 w/v histamine (positive control) and diluent (negative control) were injected intradermally. After 20 minutes, test-sites were scored from 0 to 4 compared to the controls. Reactions ≥ 2 were considered positive (Nuttall et al 1998).

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

Sample collection

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

Allergen extracts

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

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Ten microlitres of each extract was diluted 1 : 1 in either reducing (0·125 M tris/HCl, 4 per cent SDS, 20 per cent glycerol, 0·005 per cent bromophenol blue and 10 per cent 2-mercaptoethanol) or non-reducing (without 2-mercaptoethanol) loading buffer. SDS-PAGE was performed on 10 per cent tris-glycine resolving gels according to the manufacturers recommendations (Mini-Protean II™; Biorad, Hercules, USA). Duplicate gels were stained with 2·5 per cent Coomassie blue and silver, transilluminated with white light and photographed (The Imager; Appligene Oncor, Chester-le-Street, UK).

TABLE 1: Source of house dust mite extracts

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

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

Western blots

SDS-PAGE was performed using a single well across the width of the gel with 200 µg of the *D farinae*, *D pteronyssinus* and *D microceras* extracts, and 50 µg of the purified group 1 and 2 extracts in reducing and non-reducing loading buffer. Proteins were transferred to PVDF membranes (Immobilon-P; Millipore, Bedford, USA) using 0·3 M tris-HCl/20 per cent methanol (pH 10·4) lower anode, 25 mM tris-HCl/20 per cent methanol (pH 10·4) upper anode and 25 mM tris-HCl/40 mM 6-amino-n-hexonic acid/20 per cent methanol (pH 9·4) cathode buffers according to the manufacturers instructions (Transblot SD; Biorad). Membranes were dried and stored at 4 °C.

Immunoblotting

The molecular weight markers (Biorad broad range) and a strip to identify protein bands were removed and stained with 2·5 per cent Coomassie blue.

The membranes were blocked in 5 per cent skimmed milk/tris-buffered saline (TBS) for 1 hour at room temperature, briefly rinsed in TBS, then placed in an MN28 Miniblotter (Immunectics, Cambridge, USA). Appropriate channels were incubated for 1 hour at room temperature with 85 µl of a 1/5 dilution of serum, then 85 µl of either a 1/500 dilution of mouse monoclonal (D9#31116; D. J. Deboer, University of Wisconsin) (Mab) or a 1/100 dilution of goat polyclonal (A40–125; Bethyl Laboratories, Montgomery, USA) (Pab) anti-canine IgE. Blanks and control channels with no serum or no anti-canine IgE were included in each assay. Channels were washed with 10 ml of 0·05 per cent tween 20/TBS (TTBS) after each step. The miniblotter was then dismantled and the membranes incubated at room temperature for 1 hour with 20 ml of horseradish peroxidase-conjugated bovine monoclonal anti-mouse IgG (MCA1421; Serotec, Kidlington, UK) or mouse monoclonal anti-goat IgG (GT-34; Sigma, Poole, UK) diluted 1/1000 and 1/5000 respectively. The membranes were washed 3 times for 5 minutes in TTBS, then twice for 5 minutes in TBS. The protein surface of each membrane was covered with luminol solution (ECL; Amersham-Pharmacia Biotech, Little Chalfont, UK) for 1 minute and each blot imaged (IS440CF; Kodak, Rochester, USA).

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

Data analysis

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

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

RESULTS

Intradermal skin tests

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

SDS-PAGE of HDM extracts

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

Immunoblotting

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

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

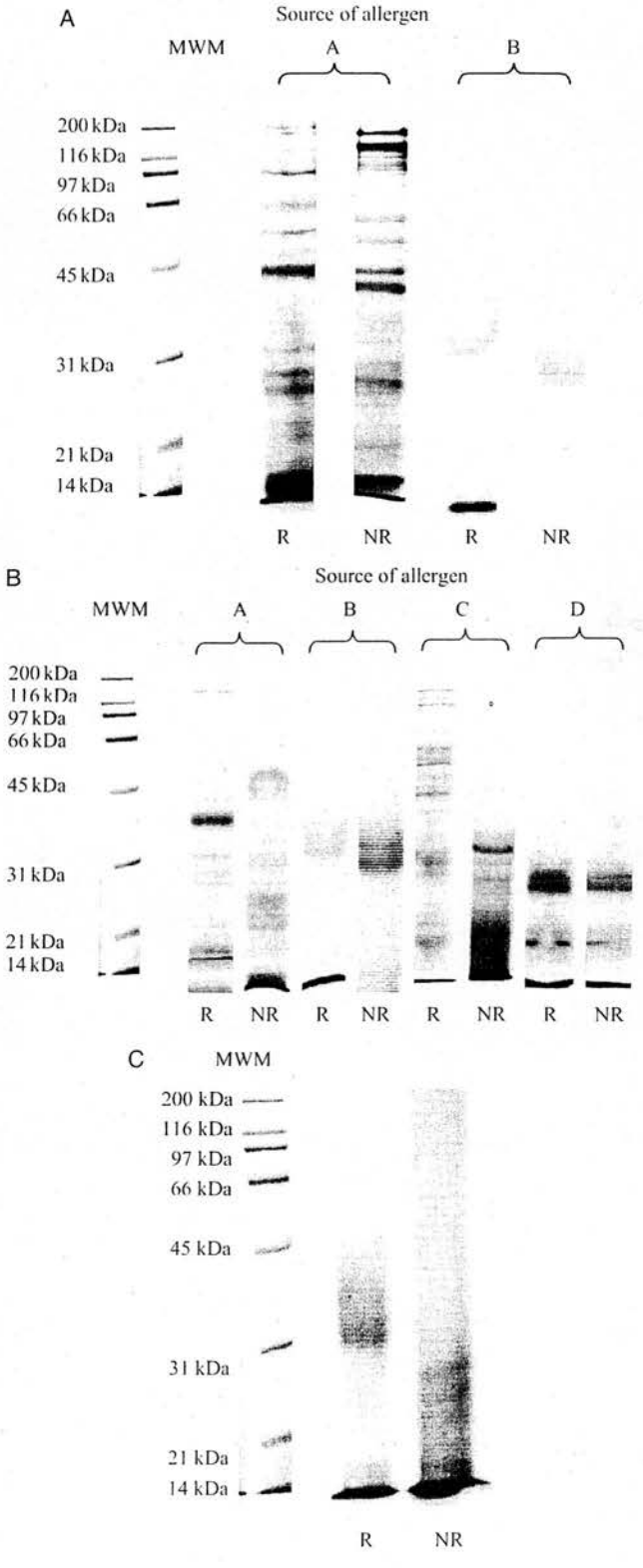

FIG 1: SDS-PAGE of crude and purified *Dermatophagoides* extracts (2·5 per cent Commassie blue stain) A – *D farinae*; B – *D pteronyssinus*; C – *D microceras*; D – Group 1 allergens; E – Group 2 allergens; MWM – Molecular weight markers (broad range; Biorad, Hercules, USA); R – Reducing buffer; NR – Non-reducing buffer; Source of allergens: A – Greer Laboratories, Lenoir, USA; B – ALK-Abéllo, Horsholm, Denmark; C – Allergo-Pharma, Hamburg, Germany; D – Rayne Laboratory, University of Edinburgh, UK.

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

Allergen extract	Positive	Negative
Dermatophagoides farinae	24	9
D pteronyssinus	22	11

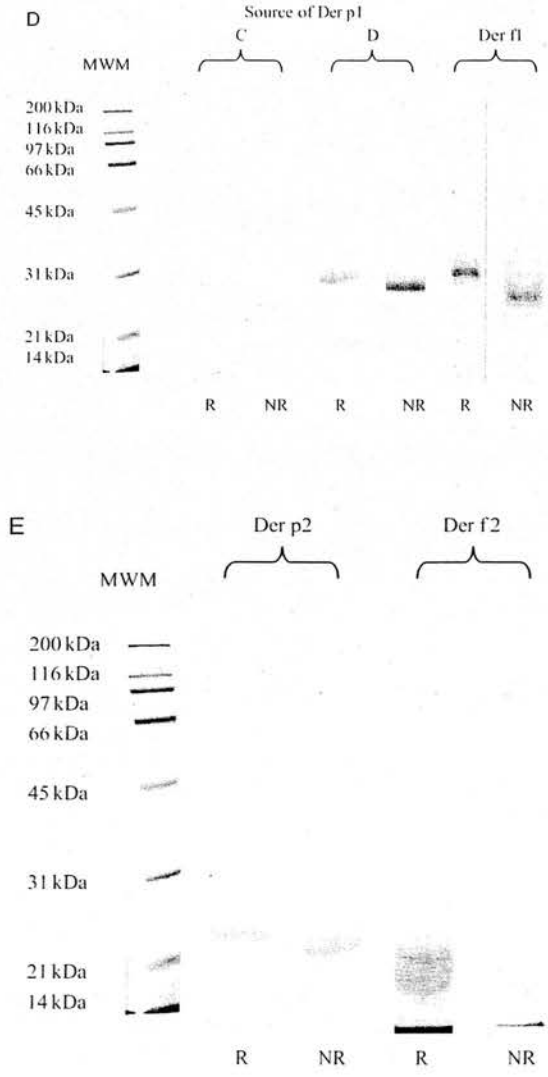

FIG 1: Continued.

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

IgE binding to D farinae *extracts*

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

IgE binding to the D pteronyssinus *extracts*

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

IgE binding to the D microceras *extract*

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

IgE binding to the purified Group 1 and 2 allergens

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

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

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

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

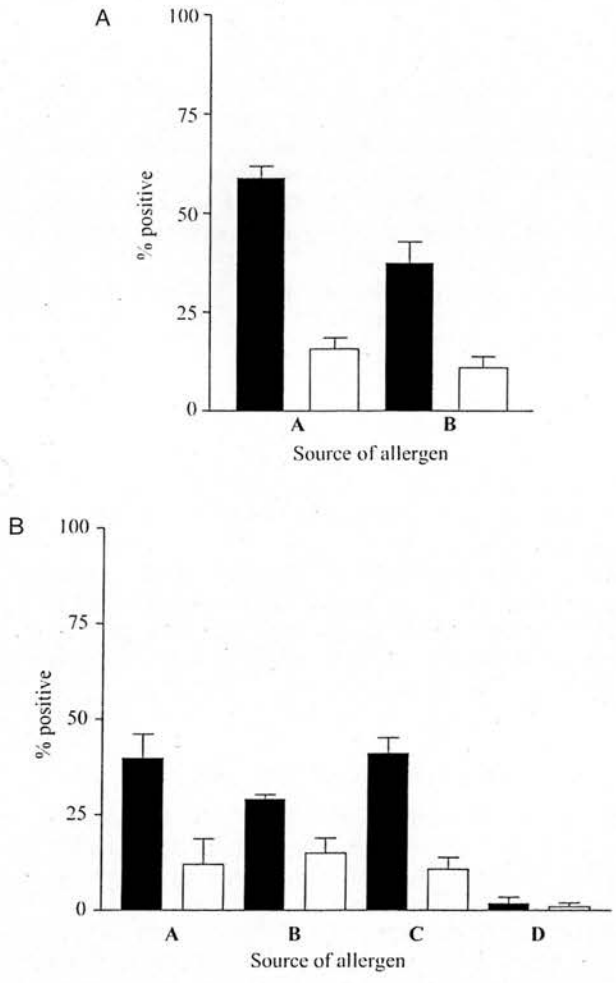

FIG 2: Percentage of atopic and healthy sera that bound to Western blots of *Dermatophagoides* allergens A – *D farinae*; B – *D pteronyssinus*; Bar - sem; ■ Atopic sera; □ Healthy sera.

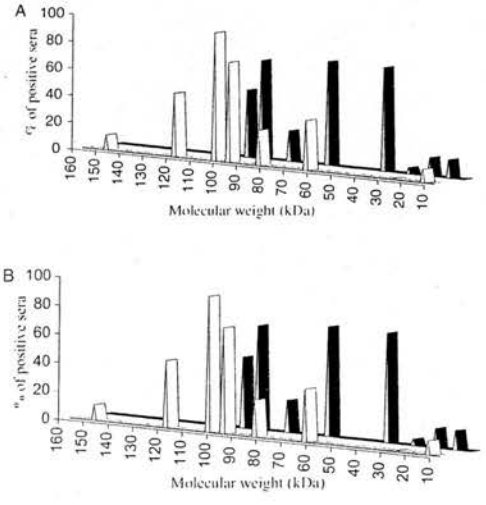

FIG 3: Major and minor allergens in the Greer *Dermatophagoides* extracts (bars greater than 50 per cent indicate major allergens). A – *D farinae*; B – *D pteronyssinus*; ■ – Atopic sera; ▒ – Healthy sera.

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

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

Major and minor allergens

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

DISCUSSION

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

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

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

To our knowledge, this is the first study using anti-canine IgE to demonstrate that different *Dermatophagoides* allergens are important in human and canine AD. This is surprising, as many allergens have intrinsic allergenic properties (Huby et al 2000). In particular, Der p1 degrades tight junctions, cleaves α1-antitrypsin, CD23 and CD25, which directly activates mast cells and basophils, inhibits T_{H1} differentiation and increases epithelial permeability, protease activity, IgE and inflammatory mediator release (Schulz et al 1999, Shakib and Gough 2000, Wan et al 2000). The 98 kDa chitinase is glycosylated, which enhances IgE binding and uptake by antigen presenting cells (APCs) (Huby et al 2000), but it is unknown if any of the allergens identified with canine sera are biologically active. In vivo activity could also be substrate and species specific. Furthermore, unlike B-cells, T-cells recognise short sequences of 10–15 amino acids associated with class II MHC molecules (Renz 1995). These are derived from allergen processed by APCs (Carrasco-Marín et al 1998), which profoundly influence the subsequent immune responses (Maekawa et al 1998). If canine APCs present a different array of peptides or if T-cell receptor and MHC class II repertoires are very distinct from humans, the specificity of allergen recognition could also differ.

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

Recognition of the *D farinae* extracts by atopic sera was influenced by the allergen source. However, there was no difference in binding between the *D pteronyssinus* extracts, despite a similar variation in protein content. Moreover, differences in binding between *D farinae* and *D pteronyssinus* were restricted to a single allergen source. The concentration of key allergens in certain extracts could explain why *D farinae*, despite being less common than *D pteronyssinus* in the UK (Thomas et al 1998), is a more prominent allergen in canine AD (Sture et al 1995). However, a recent study speculated that an apparently low prevalence of *D farinae* could be due to seasonal fluctuations, as equal levels of Der f1 and Der p1 were present in house dust (Sopelete et al 2000). Further work to define and standardise the allergen content of *Dermatophagoides* extracts is needed.

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

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

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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.

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 poly-sucrose/sodium diatrizoate solution (Histopaque®-1077; Sigma, Poole, UK) and centrifuged at 440 × *g* for 20 min at 20°C. Cells were collected from the interface, washed in a 10× volume of chilled citrated PBS and centrifuged at 250 × *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× PBS. The cell suspension was then washed in a 3× volume of chilled citrated PBS and centrifuged at 200 × *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× 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 haemacytometer 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% CO_2 for 7 days. Methyl-^{3}H 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

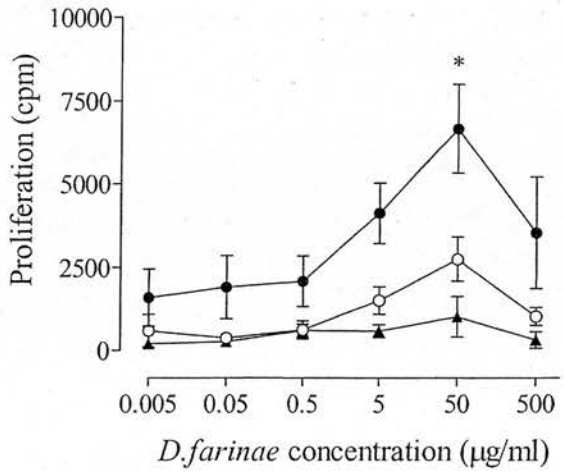

Fig. 1. PBMC responses to *D. farinae* in atopic and healthy dogs: (●) dogs with atopic dermatitis skin test positive to *D. farinae* ($n = 10$); (○) dogs with atopic dermatitis skin test negative to *D. farinae* ($n = 7$); (▲) healthy dogs ($n = 10$); (*) $P < 0.05$; bar, S.E.M.; cpm, counts per min (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 TGFβ 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	*Bordetella*	Parvovirus
Dermatophagoides farinae positive atopic dogs ($n = 10$)	1909 (1848)	49935 (23178)	5736 (6535)	3975 (4485)
Dermatophagoides farinae 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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Peripheral blood mononuclear cell responses to major and minor *Dermatophagoides* allergens 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. 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.

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

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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 chromotography (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 440*g* for 20 min at 20 °C. Cells were collected from the interface, washed in a 10× volume of chilled citrated PBS and centrifuged at 250*g* 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× PBS. The cell suspension was then washed in a 3× volume of chilled citrated PBS and centrifuged at 200*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 μM 2-mercapto-ethanol (Life Technologies), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B (100× 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 haemacytometer 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% CO_2 for 7 days. 0.5 μCi methyl-^{3}H 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; Wallac, Turku, Finland) and the activity of each well recorded as counts per minute (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; 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
D. farinae	11850 (4787; 13)	2431 (3392; 3)
D. pteronyssinus	13861 (9085; 13)	2961 (2922; 3)
D. microceras	4208 (3774; 13)	1834 (2473; 3)
98/104 kD *D. farinae* 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)
B. bronchseptica	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 α1-antitrypsin, CD23 and CD25, which directly activates mast cells and basophils, inhibits T_{H1}

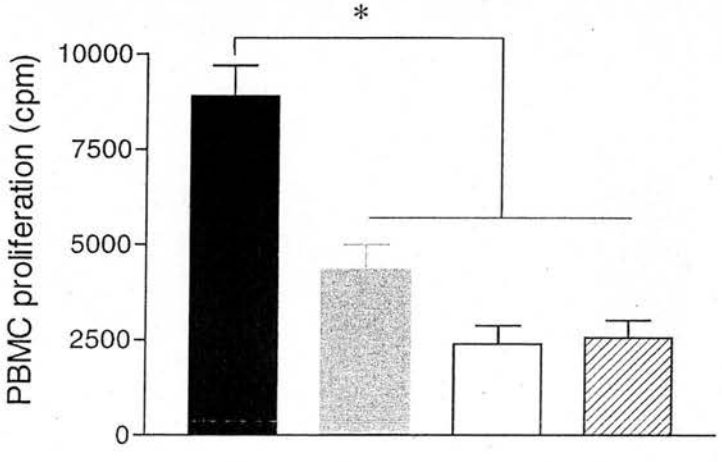

Fig. 1. Responses of PBMCs isolated from dogs with atopic dermatitis to purified *D. farinae* allergens: (■) 98 kD allergen ($n = 7$ dogs); (▤) Der f 1 ($n = 11$ dogs); (□) Der f 2 ($n = 11$ dogs); (▨) resting cells ($n = 11$ dogs); bar: S.E.M.; *: $p < 0.05$; cpm: counts per minute, expressed as mean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

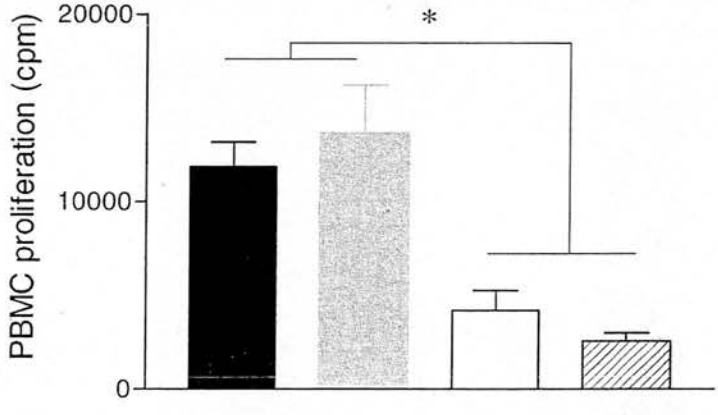

Fig. 2. Responses of PBMCs isolated from dogs with atopic dermatitis to crude *Dermatophagoides* extracts: (■) *D. farinae* ($n = 13$ dogs); (□) *D. pteronyssinus* ($n = 13$ dogs); (□) *D. microceras* ($n = 13$ dogs); (▨) resting cells ($n = 13$ dogs); bar: S.E.M.; *: $p < 0.05$; cpm: counts per minute,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

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Expression of Th1, Th2 and immunosuppressive cytokine gene transcripts in canine atopic dermatitis

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Summary

Background Atopic dermatitis is a common inflammatory skin disease of humans and dogs. Human atopic dermatitis is associated with Th2-type responses, although Th1 cytokines can be identified in chronic lesions. In contrast, tolerance to environmental allergens in healthy individuals is mediated by regulatory T cells.

Objective This study examined the expression of the immunosuppressive cytokines TGF-β and IL-10, the Th2-type cytokines IL-4 and IL-6, and the Th1-type cytokines IFN-γ, TNF-α, IL-2, IL-12p35 and IL-12p40, in canine atopic dermatitis.

Materials and methods RNA was isolated from lesional atopic, non-lesional atopic and healthy canine skin samples. Semi-quantitative reverse transcriptase polymerase chain reactions (RT-PCRs) were carried out using specific primers and one-way analyses of variance used to compare cytokine expression in each group.

Results Canine atopic dermatitis was associated with over-expression of IL-4 mRNA and reduced transcription of TGF-β compared with healthy skin ($P < 0.05$). Higher levels of IFN-γ, TNF-α and IL-2 mRNA were seen in lesional compared with non-lesional and healthy skin ($P < 0.05$). There were no significant differences in IL-10, IL-6, IL-12p35 or IL-12p40 transcription between the three groups.

Conclusions This is the first report to demonstrate that canine atopic dermatitis is associated with over-production of IL-4. Clinical tolerance in healthy individuals appears to be associated with TGF-β, although it is unclear if this reflects an active mechanism or simply non-responsiveness of the immune system. Th1 cytokines may be induced by subsequent self-trauma and secondary infections in atopic skin. We believe that these results better characterize spontaneously occurring canine atopic dermatitis. We further propose that this should be investigated as a possible animal model of human atopic dermatitis.

Keywords atopic dermatitis, canine, cytokines, interleukin 4, Thelper 1, Thelper 2, transforming growth factor beta, T regulatory cells

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Introduction

Atopic dermatitis is a universally recognized, chronic inflammatory skin disease of humans [1] and dogs [2]. The incidence of atopic dermatitis in humans has increased dramatically in the last 30 years [1]. It is difficult to assess whether the incidence of canine atopic dermatitis has increased to the same extent, as the development of diagnostic techniques in veterinary dermatology may have increased recognition of the condition, but some authors estimate that up to 10% of all dogs are now affected [2].

Recent interest has focused on the role of $CD4^+$ T helper (Th) cells in human atopic dermatitis [3]. Th1 cells were first differentiated from Th2 cells in rats and mice [4, 5]. Unlike rodent Th cells, human Th cells secrete a complex variety of cytokines [6], although Th1- and Th2-type populations can be distinguished [7]. Th1-type cytokines, including IFN-γ), IL-2, IL-12 and TNF-α, promote cell-mediated immunity, whereas the Th2-type cytokines IL-4, IL-5, IL-6, and IL-13, promote humoral immunity, including IgE production [6, 8].

Several studies have detected Th2 polarization in peripheral blood mononuclear cells and skin of atopic humans [9–13] and mouse models of atopic dermatitis [14, 15]. Th2 and Th1 responses are regarded as mutually exclusive [16], but Th1-type cytokines have been detected in chronic lesions [17] and dominate atopy patch test sites after 48 h [18], suggesting they also participate in pathogenesis of chronic atopic dermatitis. Furthermore, healthy individuals do not develop Th1-induced cell-mediated inflammatory reactions to environmental allergens [19]. Their lack of responsiveness may be mediated by the immunosuppressive cytokines IL-10 and TGF-β [20, 21], although clinical tolerance could also result from immune

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non-responsiveness or a failure of environmental allergens to penetrate healthy skin.

Studies of cytokine expression in canine atopic dermatitis are limited. IL-4 has been detected in lesional atopic skin by immunohistochemistry [22]. Another study using non-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) demonstrated a Th2-like cytokine pattern in 25% of atopic and a Th1-like pattern in 25% of healthy skin samples [23].

In this study we used semiquantitative RT-PCR to compare mRNA expression of Th1-type (IFN-γ, TNF-α, IL-2 and IL-12), Th2-type (IL-4 and IL-6), and immunosuppressive cytokines (IL-10 and TGF-β) in atopic and healthy canine skin. Our aim was to determine if canine atopic dermatitis was associated with polarized Th1/Th2 responses similar to those observed in human diseases. This information would greatly increase our understanding of the pathogenesis of the canine disease, and would help characterize this spontaneously occurring condition as well as being informative about its applicability to human atopic dermatitis.

Materials and methods

Study population

Dogs with atopic dermatitis ($n = 26$) 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 [24, 25]. Coat brushings, skin scrapings and trial therapy were used to eliminate the possibility of an ectoparasite infestation. All dogs underwent a 6-week 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 *Dermatophagoides farinae* and *D. pteronyssinus* (Greer Laboratories, Lenoir, NC, USA), as follows. Dogs were sedated with 0.15 mg/kg xylazine injected intramuscularly. Hair was clipped from the lateral flank and 0.05 mL of each allergen extract, 0.1 mg/mL histamine (positive control) and saline with phenol diluent (negative control) were injected intradermally. Test sites were assessed after 20 min according to standard criteria and subjectively scored from 0 to 4 compared with the controls. By convention, reactions greater than 2 were considered positive [25].

Control samples were taken from healthy dogs ($n = 12$) presented for euthanasia at the University of Edinburgh Hospital for Small Animals with no history and clinical signs of pruritus or conditions likely to alter immune function.

Sample collection

Skin samples were taken whilst the dogs were sedated as follows: 0.5–1.0 mL of 1% lignocaine was infiltrated into the subcutis under the sites marked for biopsy. Non-lesional samples ($n = 26$) were taken from the flank. Lesional samples ($n = 12$) were taken from areas of erythema and macular-papular dermatitis, where present. Grossly evident excoriation, staphylococcal pyoderma and *Malassezia* dermatitis were avoided. Six-millimetre diameter skin biopsies were taken and placed immediately into RNA-later® (Ambion Inc., Austin, TX, USA). Skin ($n = 12$) and positive control samples (popliteal lymph node and tonsil) from healthy dogs were collected into RNA-later® immediately after euthanasia. Samples in RNA-later® were kept at 4 °C for 24 h, before storage at − 20 °C.

Primer design

Primers (Table 1) were designed from published canine sequences (Genbank®; National Center for Biotechnology Information; URL, http://www.ncbi.nlm.nih.gov/Genbank/) using Oligo6® software (Molecular Biology Insights, Cascade, CO, USA). Cross-reactive oligonucleotides were eliminated by screening against known sequences (BLAST®; National Centre for Biotechnology Information; URL, http://www.ncbi.nlm.nih.gov/BLAST/).

RNA extraction

Total RNA was extracted from the skin biopsies and control material by homogenization (Ultra-turrax 125; Janke and Kunkel IKA, Staufen, Germany) in TRI-reagent® (Sigma, Poole, UK) [26, 27]. Genomic deoxyribonucleic acid was removed by incubating with 4IU DNAse I per 50 μL RNA solution according to the manufacturers instructions (DNA-free®; Ambion Inc.). The RNA concentration was quantified by ultra-violet absorbance at 260 nm with a 260-nm : 280 nm ratio greater than 1.5 (DU650 spectrophotometer; Beckman-Coulter, Fullerton, CA, USA). Heparin was removed by incubating 4 μg RNA with 4IU heparinase I (Sigma) [28]. Heparinazed RNA was stored at − 70 °C.

RT-PCR

cDNA generated from 1 μg heparinazed RNA (Reverse Transcriptase System; Promega Corp., Madison, WI, USA) was diluted to 200 μL in RNAse free water; 1 μg of heparinazed RNA was diluted to 200 μL without reverse transcription as a negative control. cDNA and negative control preparations were stored at − 20 °C.

PCRs were carried out in 50-μL volumes containing 10 mM Tris-HCl, 2.5IU *Taq* deoxyribonucleic acid polymerase (Roche Molecular Biochemicals, Lewes, UK), 200 μM deoxynucleotide triphosphates, 200 nM of each primer pair and 50 ng cDNA. Reaction conditions consisted of initial denaturing at 94 °C for 2 min, then 35 cycles of denaturing at 94 °C for 30 s, annealing for 30 s and elongation at 72 °C for 1 min, before a final elongation at 72 °C for 7 min (GeneAmp PCR System 2400; Perkin-Elmer, Cambridge, UK). Optimum $MgCl_2$ concentration and annealing temperatures for each primer pair were established prior to the study (Table 1). PCR products were run on 1.2% agarose gels containing 0.5 μg/mL ethidium bromide and imaged under 590 nm ultra-violet light (Image Station 400CF; Kodak, Rochester, NY, USA). PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primers was performed for each cDNA and negative control preparation. Samples with no GAPDH signal or a positive signal from a negative control preparation were discarded.

Southern blotting and hybridization

PCRs for each primer pair using positive control cDNA (derived from popliteal lymph node and tonsil) were performed as described previously. Southern blotting and hybridization was performed using specific digoxigenin-labelled internal oligonucleotides [26].

Table 1. Cytokine primers and optimum conditions for polymerase chain reactions

Cytokine	Primer sequence	Annealing temperature(°C)	$MgCl_2$ (mM)	pH	PCR cycles	Product size(bp)
GAPDH	Forward – CCTTCATTGACCTCAACTACAT Reverse – CCAAAGTTGTCATGGATGACC Internal – CCCTCAAGATTGTCAGCAATGCC	55	2.0	8.6	35	400
IL-4	Forward – TAAAGGGTCTCACCTCCCAACTG Reverse – TAGAACAGGTCTTGTTTGCCATGC Internal – CACCAGCACCTTTGTCCACGGA	55	1.5	9.2	36	317
IL-6	Forward – CCACAAGCGCCTTCTCCCTGG Reverse – TCACGCACTCATCCTGCGACTG Internal – GAATCACTACCGGTCTTGTGGAG	60	1.0	8.3	35	532
IFN-γ	Forward – TCGGACGGTGGGTCTCTTTTCG Reverse – CACTTTGATGAGTTCATTTATCGCC Internal – CAGCACCAGTAAGAGGGAGGAC	60	2.5	8.9	35	281
IL-2	Forward – CTCACAGTAACCTCAACTCCTGC Reverse – TTCTGTAATGGTTGCTGTCTCGTC Internal – ACACGCCCAAGAAGGCCACAGA	55	2.5	8.6	35	461
TNF-α	Forward – ACTCTTCTGCCTGCTGCACTTTGG Reverse – GTTGACCTTTGTCTGGTAGGAGACGG Internal – CACCCACACCATCAGCCGCTTCGCCG	55	2.5	8.9	35	366
IL-12p35	Forward – AGCCTCACCGAGCCCAGGA Reverse – TAAGGCACAGGACCGTCATAAAAG Internal – TGGAGGCCTGCTTACCACTGGA	60	2.0	8.6	34	293
IL-12p40	Forward – GCCCCCGGAGAAATGGTGGTC Reverse – TTGTGGCACACGACCTTGGCTG Internal – ACCAGACCCACCCACAAACCTG	65	1.5	8.3	34	783
TGF-β	Forward – AGTTAAAAGCGGAGCAGCATGTGG Reverse – GATCCTTGCGGAAGTCAATGTAGAGC Internal – GTTCAGTTCCAGCCCGCCGAGGT	55	2.5	9.2	35	434
IL-10	Forward – GTCCCTGCTGGAGGACTTTAAGA Reverse – TGGTCGGCTCTCCTACATCTCG Internal – AAGGCGGTGGAGCAGGTGAAGA	55	1.5	8.6	35	443

Semi-quantitative PCR

To determine the optimum number of cycles for semiquantitative PCR, 26–44 PCR cycles for each primer pair were performed using positive control cDNA and the gels imaged as described. Standard curves were generated by plotting the number of PCR cycles against the log2 net band intensity. The optimum number of cycles for each cytokine primer pair was selected from the straight-line portion of the plot (Table 1).

PCRs for each cytokine primer pair were performed with cDNA from each skin sample for the appropriate number of cycles. Positive control cDNA and negative controls (water only) were included in each PCR. PCR products were run on gels and imaged as before. Gels with no positive control signal or a negative control signal were discarded. The relative abundance of cytokine gene transcripts was determined by dividing the cytokine PCR product signal intensity by that of GAPDH for each skin sample.

Data analysis

After ensuring data were normally distributed, one-way analyses of variance with Tukey's post-tests (Instat; Graphpad Inc., San Diego, CA, USA) were used to compare the relative abundance of cytokine gene transcripts in healthy, lesional atopic and non-lesional atopic skin. The level of significance was set at $P < 0.05$.

Results

All the dogs with clinical diagnosis of atopic dermatitis had positive skin tests to *D. farinae* and *D. pteronyssinus*. There were also a number of individual reactions to a range of other dust mites, epithelia, pollens and moulds. Single bands of the expected size for each cytokine were seen on the PCR gel and hybridization blots using positive control material. This verified expression of mRNA for each cytokine in the positive control samples and confirmed primer specificity (Fig. 1).

Expression of Th2-type cytokines

Expression of IL-4 mRNA was largely restricted to atopic skin. Significantly higher levels of IL-4 mRNA were detected in both lesional and non-lesional atopic skin compared with healthy skin ($P < 0.05$) (Fig. 2a), although there was no significant difference in transcription between lesional and non-lesional atopic skin. IL-6 mRNA expression was also greatest in atopic skin (Fig. 2b). However, IL-6 was also expressed in healthy skin and differences between the three groups failed to reach significance.

Expression of Th1-type cytokines

There was a similar pattern of transcription for the Th1-type cytokines IFN-γ (Fig. 2c), IL-2 (Fig. 2d) and TNF-α (Fig. 2e) in

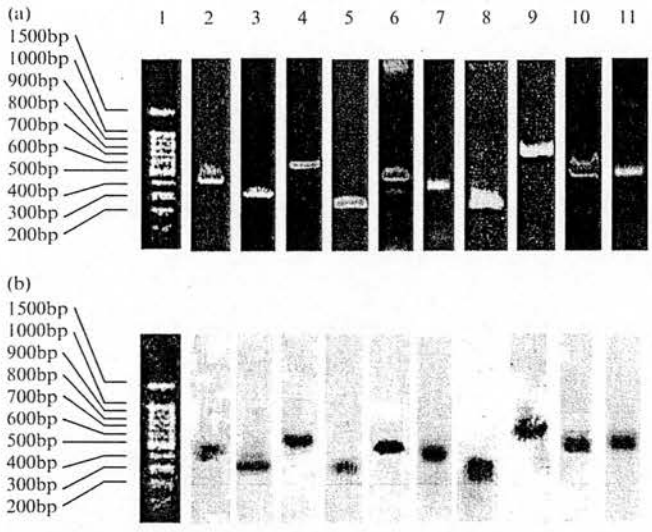


Fig. 1. Detection of cytokine gene transcription using positive control cDNA (Image Station 440CF; Kodak). (a) 1.2% agarose gel with 0.5 μg/mL ethidium bromide. (b) Southern blotting and hybridization to digoxigenin labelled internal probes. Lane 1 = 100 bp DNA markers (Promega Corp.); lane 2 = GAPDH; lane 3 = IL-4; lane 4 = IL-6; lane 5 = IFN-γ; lane 6 = IL-2; lane 7 = TNF-α; lane 8 = IL-12p35; lane 9 = IL-12p40; lane 10 = TGF-β; lane 11 = IL-10.

all three groups of samples. Significantly higher levels of mRNA for each cytokine were detected in lesional atopic skin compared with either non-lesional atopic or healthy skin ($P < 0.05$). Variably low levels, which did not significantly differ, were detected in non-lesional and healthy skin.

Levels of IL-12p35 and IL-12p40 mRNA were highly variable in both atopic and healthy skin. IL-12p35 transcription did not appear to differ between lesional atopic, non-lesional atopic and healthy skin (Fig. 2f). IL-12p40 transcription was highest in lesional atopic and healthy skin but there was no significant difference between the three groups of samples (Fig. 2g).

Immunosuppressive cytokines

Healthy skin was associated with expression of mRNA for the immunosuppressive cytokine TGF-β. Levels of TGF-β mRNA were significantly higher in healthy compared with atopic skin (Fig. 2h). There was no significant difference between the lower levels of expression in lesional and non-lesional atopic skin.

Levels of mRNA for IL-10 were highly variable and no significant difference was detected between any of the three groups (Fig. 2i).

Discussion

Studies in humans and experimental mouse models suggest that IL-4 expression is a hallmark of atopic dermatitis [3]. In dogs, a non-quantitative study detected IL-4 transcripts in both atopic and healthy skin [23]. However, using semiquantitative RT-PCR, this study has shown that much higher levels of IL-4 mRNA are expressed in atopic compared with healthy canine skin. This demonstrates for the first time that IL-4 may contribute to the pathogenesis of canine atopic dermatitis. The high levels of IL-4 mRNA expression found in non-lesional as well as lesional atopic skin in this study are consistent with the pattern of transcription in human atopic dermatitis [3]. It is not clear, however, if IL-4 expression in non-lesional skin is constitutive or is a response to specific allergens. The impaired delayed-type hypersensitivity responses seen in atopic humans and animals are evidence of a default Th2 response [29]. However, Th1 and Th2 responses can also coexist in the same individual, suggesting that Th1 and Th2 responses are antigen-specific [30].

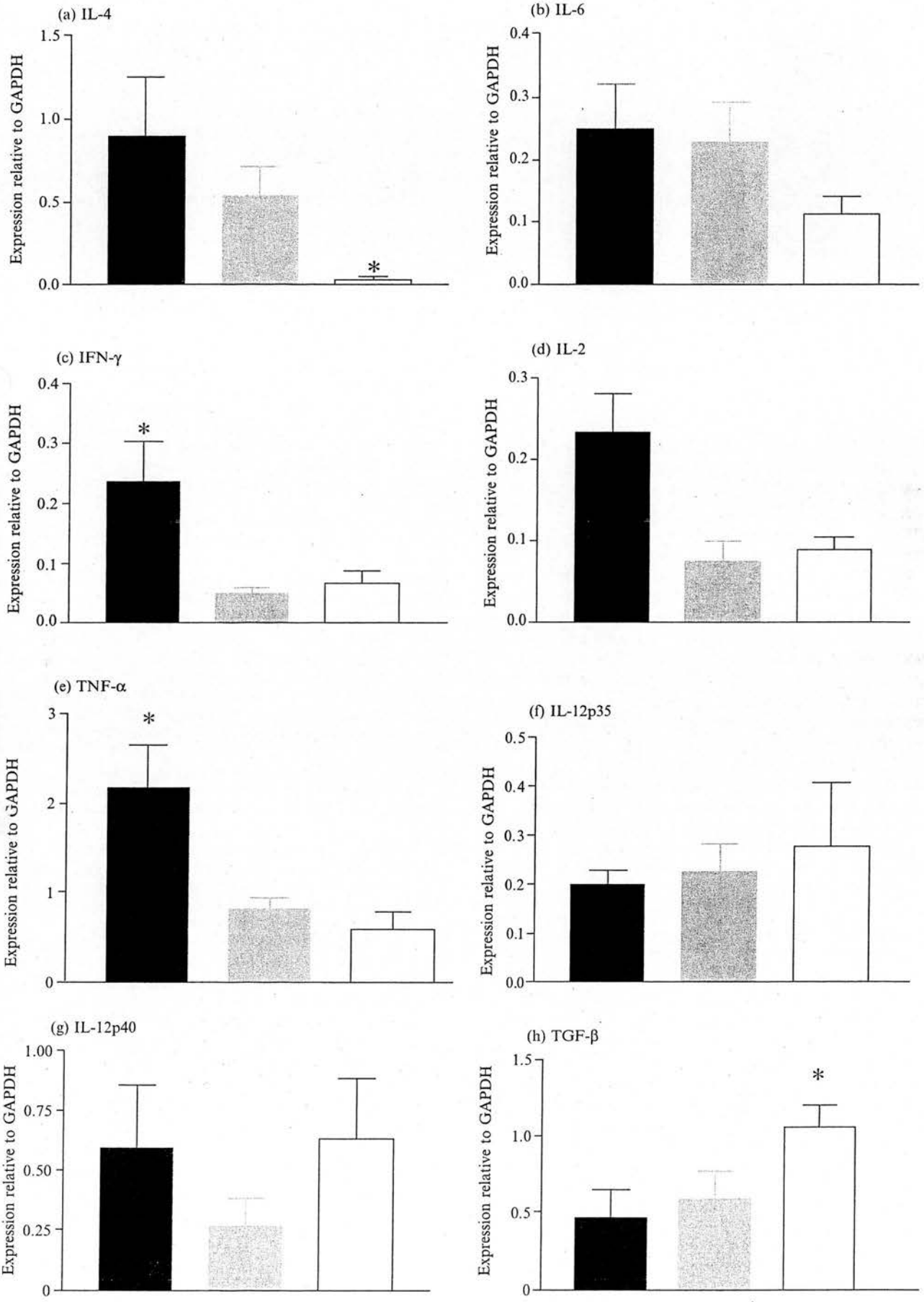
In contrast to a previous investigation [23], IL-6 mRNA was readily detected in this study. However, there was no significant difference in the level of expression between atopic and healthy canine skin. Although IL-6 is regarded as a Th2-type cytokine [7], it is also produced by antigen-presenting cells, fibroblasts, macrophages, endothelial cells and keratinocytes [31], and its role may depend on the precise relationship between and state of activation of these different cell types. Furthermore, IL-6 may support production of the allergen-specific IgG found in healthy humans and dogs [32, 33].

Here we report for the first time that much lower levels of TGF-β mRNA are expressed in canine atopic dermatitis compared with healthy skin. TGF-β, produced by antigen-presenting cells, T cells and keratinocytes, is thought to be important in peripheral and mucosal tolerance to environmental allergens in humans and rodent models [34, 35]. Atopic dermatitis in humans and dogs is also associated with hyper-reactivity to irritant stimuli [2, 3], which could be explained in part by low TGF-β expression. In contrast, constitutive expression of TGF-β in healthy skin would inhibit inflammatory responses to environmental allergens. However, TGF-β is secreted in an inactive form and is subject to post-transcription regulation [36]. Confirmation of its importance awaits demonstration of active TGF-β in canine skin.

Tolerance may also be mediated by IL-10. Although an earlier investigation failed to demonstrate its presence in canine skin [23], IL-10 transcripts were present in all the samples in this study. There was, however, no difference in the level of expression between atopic and healthy skin. The role of IL-10 is unclear in human disease and rodent models. Decreased IL-10 expression has been associated with colitis and cutaneous inflammation in mice [37], and atopic dermatitis in children [12]. However, increased IL-10 expression has also been found in Th cells from patients with atopic dermatitis [38] and in the airways and skin after allergen challenge in mice and humans [21,39]. IL-10 is constitutively expressed in normal mouse skin, but only in late phase reactions in a model of atopic dermatitis [40], which may fail to resolve established Th2 responses and allergen-induced inflammation [20, 37].

The Th1-type cytokines IL-2, IFN-γ and TNF-α are associated with cell-mediated inflammation and inhibition of Th2 differentiation [16]. An earlier non-quantitative investigation detected IL-2 transcripts more frequently in healthy than atopic canine skin samples, although there was no difference in the number of samples that expressed IFN-γ and TNF-α [23]. In contrast, only low levels of IL-2, IFN-γ and TNF-α mRNA were detected in healthy canine skin in this study. These results are not consistent with Th1 polarization in healthy skin, although it would be unusual to find high levels of pro-inflammatory cytokines expressed in healthy skin.

High levels of mRNA for the Th1 cytokines IL-2, IFN-γ and TNF-α were restricted to lesional atopic skin. This suggests that


(a) IL-4
Expression relative to GAPDH
1.5
1.0
0.5
0.0
*
(b) IL-6
Expression relative to GAPDH
0.4
0.3
0.2
0.1
0.0
(c) IFN-γ
Expression relative to GAPDH
0.4
0.3
0.2
0.1
0.0
*
(d) IL-2
Expression relative to GAPDH
0.3
0.2
0.1
0.0
(e) TNF-α
Expression relative to GAPDH
3
2
1
0
*
(f) IL-12p35
Expression relative to GAPDH
0.5
0.4
0.3
0.2
0.1
0.0
(g) IL-12p40
Expression relative to GAPDH
1.00
0.75
0.50
0.25
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(h) TGF-β
Expression relative to GAPDH
1.5
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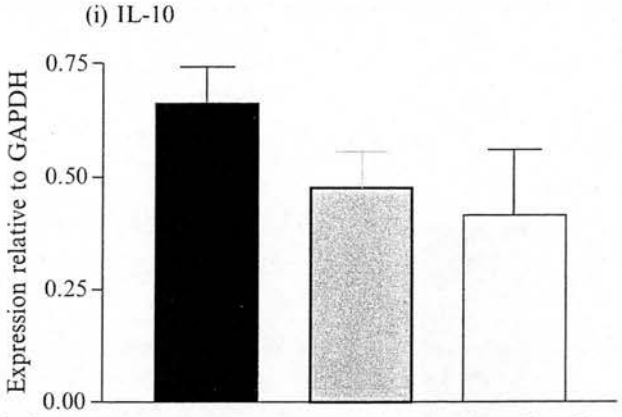

Fig. 2. Abundance of cytokine gene transcripts in atopic and healthy canine skin. Results of densitometric analysis (Image station 440CF; Kodak) of cytokine gene transcription as measured by RT-PCR. Data are expressed as arbitrary units relative to GAPDH. ■ = lesional atopic dermatitis; □= non-lesional atopic dermatitis; □ = healthy skin; bar = standard error of the mean; $^*P < 0.05$. a = IL-4; b = IL-6; c = IFN-γ; d = IL-2; e = TNF-α; f = IL-12p35; g = IL-12p40; h = TGF-β; I = IL-10.

Th2 skewing in canine atopic dermatitis is not associated with poor expression of Th1 cytokines. Chronic lesions in human atopic dermatitis also exhibit a mixed pattern of cytokines, including IL-4, IL-13, IL-5, IL-2, IFN-γ and IL-12 [17]. Furthermore, Th1 cytokines dominate atopy patch tests after 48 h [18, 41]. Why Th1 cytokines are expressed in chronic atopic dermatitis is not fully understood. Chronic lesions include papules, scaling, crusting and lichenification [1, 2], which may result from self-trauma and microbial colonization rather than atopic dermatitis *per se*. Self-trauma induces inflammatory cytokines, including IL-1, TNFα and granulocyte-macrophage colony-stimulating factor from keratinocytes [42]. Furthermore, staphylococcal superantigens induce cutaneous lymphocyte antigen expression on T-cells and IL-1, TNFα and IL-12 from mononuclear cells in humans [3, 43].

IL-12 is a critical factor in Th1 differentiation, IFNγ production and cutaneous lymphocyte antigen expression [44, 45]. Down-regulation of IL-12 has been associated with Th2 polarization [20] but there was no significant difference between IL-12 transcripts in healthy and atopic canine skin in this study. Whilst this does not preclude post-transcription dysregulation of IL-12, the expression of Th1-type cytokines suggests that this is not the case, at least in lesional skin. IL-12 can also support established Th2 responses [46], which may lead to a vicious circle of ongoing inflammation in atopic skin.

These results allow us to postulate a pathogenic sequence for canine atopic dermatitis in which early atopic dermatitis is associated with increased expression of IL-4 and decreased expression of TGF-β. Lesional skin, in contrast, exhibits a mixed inflammatory cytokine pattern that may be due to self-trauma and secondary infection. The mechanisms underlying Th2 polarization in non-lesional skin are unclear, but do not appear to involve dysregulation of IL-12. Atopic dermatitis may also reflect a loss of peripheral tolerance to environmental allergens, which in healthy skin appears to be associated with TGF-β rather than Th1 polarization. We further propose that, as the human and canine diseases share similar characteristics, canine atopic dermatitis should be developed as a naturally occurring model of human atopic dermatitis.

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