Further studies on the immunopathogenesis of canine atopic dermatitis

by

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Declaration

I declare that all work included in this thesis is my own except where otherwise stated. No part of this work has been, or will be, submitted for any other degree of professional qualification.

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Abstract

Various aspects of the pathogenesis of canine atopic dermatitis have been elucidated such as the role of certain allergens, antigen presenting cells, allergen-specific T lymphocytes, IgE and mast cells. However, gaps in our understanding still remain, such as the role of antigen-specific IgG and cytokine subsets. Canine atopic dermatitis is commonly treated using allergen-specific immunotherapy, but the mechanisms of its action are still incompletely understood. The studies in this thesis investigate further some aspects of the immunopathogenesis of canine atopic dermatitis and the changes that occur during allergen-specific immunotherapy.

To investigate IgG responses to *D. farinae* antigens, a semi-quantitative, Western blot, digital image analysis system was developed and validated. Both healthy and atopic dogs mounted *D. farinae*-specific total IgG, IgG1 and IgG4 responses to separated antigens. *D. farinae*-specific IgG2 and IgG3 responses were difficult to detect. The profile of IgG binding was similar in the two groups, both in terms of the number of bands recognised and their molecular weights. The most commonly recognised band in both groups was a 98 kDa antigen, most likely to be the major allergen Der f 15. These results indicate that *D. farinae* antigens are recognised by the canine immune system regardless of whether or not a dog is atopic. They also demonstrate that antibody class switching to IgE in atopic dogs does not appear to inhibit IgG production. The IgG antibody response does not appear to be protective against the development of clinical atopic disease, but whether or not it plays any role in the pathogenesis of the disease remains to be determined.

In atopic dogs undergoing allergen-specific immunotherapy (ASIT) against *D. farinae* with alum-precipitated vaccines, there was no significant increase in *D. farinae*-specific total IgG, IgG1 or IgG4, even in dogs showing apparent clinical improvements. In contrast, ASIT using aqueous vaccines resulted in significant increases in *D. farinae*-specific IgG responses. These results suggest that aqueous ASIT may elicit the production of IgG blocking antibodies in atopic dogs, an effect not detected using alum-precipitated vaccines.

To investigate the cytokine milieu in dogs with atopic dermatitis, real-time quantitative RT-PCR was used to detect the expression of mRNA transcripts of the Th1 cytokine IFN-γ, the Th2 cytokine IL-4, the Treg cytokine TGF-β and inducible NO synthase (iNOS) as a measure of the innate immune response. The housekeeping gene for 18S ribosomal RNA (rRNA) was chosen as internal control to normalise variations in the amount of starting material between samples and between individuals. The expression of TGF-β and iNOS were lower in lesional skin compared to non-lesional skin in atopic dogs, whilst IFN-γ was expressed in a significantly higher level in lesional skin compared to healthy controls. IL-4 expression did not differ between the groups. These cytokine profiles show distinct differences to those reported using non real-time, semi-quantitative RT-PCR. These differences may reflect the various methodological approaches used and illustrate the limitations inherent in such techniques for the quantification of cytokine expression.

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Abbreviations

A_{260}	Absorbance as measured at 260 nm
ABPA	Allergic bronchopulmonary aspergillosis
AD	Atopic dermatitis
ADCC	Antibody-dependent cell cytotoxicity
AMV	Avian myeloblastosis virus
APC	Antigen-presenting cell
APT	Atopy patch test
ASIgES	Allergen-specific IgE serology
ASIT	Allergen-specific immunotherapy
BLAST	Best alignment search tool
bp	Base pair
BSA	Bovine serum albumin
CAD	Canine atopic dermatitis
CADESI	Canine atopic dermatitis extend and severity index
CD	Contact dermatitis
cDNA	Complementary deoxyribonucleic acid
CLA	Cutaneous lymphocyte antigen
cNOS	Constitutive nitric oxide synthase
CsA	Cyclosporin A
CTL	Cytolytic T lymphocyte
DAB	3,3'-diaminobenzidine
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DEPC	Diethyl pyrocarbonate
Der	*Dermatophagoides*
DF	*Dermatophagoides farinae*
dGTP	Deoxyguanosine triphosphate
DLA	Dog leukocyte antigen
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DP	*Dermatophagoides pteronyssinus*

ds	Double stranded
DTH	Delayed-type hypersensitivity
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
E. coli	*Escherichia coli*
EAA	Extrinsic allergic alveolitis
EDTA	Ethylene diamine tetraacetic acid
EFA	Essential fatty acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
g	gravity force
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GRE	Glucocorticoid response element
GST	Glutathione S-transferase
HDM	House dust mite
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
IDT	Intradermal test
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IT	Immunotherapy
kb	Kilobase
kDa	Kilodalton
LB	Luria-Bertani
LT	Leukotriene
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MW	Molecular weight
nt	Nucleotide
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide

NOS	Nitric oxide synthase
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCA	Passive cutaneous anaphylaxis
PCR	Polymerase chain reaction
PG	Prostaglandin
PK	Prausnitz-Küstner
ppi	Pixels per inch
psi	Pounds per square inch
PVAS	Pruritus visual analog scale
PVDF	Polyvinylidene difluoride
RAST	Radioallergosorbent test
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
SE	Staphylococcal enterotoxin
SPINK5	Serine protease inhibitor Kazal – type 5
ss	Single stranded
S-TS	Short-term sensitising
TA	Tris/acetate buffer
TAE	Tris/acetate/EDTA buffer
Taq	*Thermus aquaticus*
TARC	Thymus- and activation-regulated chemokine
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween-20
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
Th cell	Helper T cell
TLR	Toll-like receptor

T_m	Melting temperature
TNF	Tumor necrosis factor
Treg cell	Regulatory T cell
TSST	Toxic shock syndrome toxin
UV	Ultraviolet
v/v	Volume per volume
VCAM	Vascular cell adhesion molecule
w/v	Weight per volume

Chapter 1

Introduction

1.1 Introduction to atopic dermatitis in humans and dogs

Atopic dermatitis (AD) is a pruritic, chronic and relapsing inflammatory skin disease that frequently occurs in humans and dogs. The cause is usually associated with a genetic predisposition to become IgE-sensitised to allergens commonly occurring in the environment to which the majority of subjects do not produce a prolonged IgE antibody response. The concept of "atopy" (derived from the Greek atopia, meaning "different" or "out of place") originally was proposed in 1923 to include asthma and allergic rhinitis, but AD was added to the group of atopic disorders in 1933 on the basis of association of this form of eczema with asthma and allergic rhinitis. Unlike the respiratory manifestations in humans, atopic disease in dogs usually manifests as dermatitis and respiratory signs are not commonly seen.

1.1.1 Hypersensitivity and atopic dermatitis

Atopic dermatitis, also known as eczema in humans (Johansson and others 2004), is commonly seen in clinical practice. The recent interest in this allergic skin disease has been sparked by reports of its increasing prevalence (Scott and others 2001; Johansson and others 2004) and the significant adverse impact that it can have on quality of life. The mechanism of atopic dermatitis has been actively investigated and the pathophysiology involves a complex series of interactions between resident and infiltrating cells orchestrated by pro-inflammatory cytokines and chemokines; however, the pathophysiological puzzle of AD is far from being elucidated completely.

Atopic dermatitis has been classified as a type I hypersensitivity reaction which implies a role for IgE and mast cell-effector mechanisms. Two types of AD, however, have been described in humans: an extrinsic form associated with IgE-

mediated sensitisation, which affects 70-80% of patients; and an intrinsic form with normal IgE levels, which affects 20-30% of patients (Novak and Bieber 2003a). In extrinsic AD, memory T cells expressing the skin homing receptor, cutaneous lymphocyte-associated antigen (CLA), produce increased amounts of Th2 cytokines. These include IL-4 and IL-13 (Akdis and others 1997; Teraki and others 2000), which are known to induce isotype switching to IgE synthesis, as well as IL-5, which plays an important role in eosinophil development and survival (Leung and others 2004). These CLA+ T cells also produce abnormally low levels of IFN-γ, a Th1 cytokine known to inhibit Th2 cell function. Intrinsic AD is associated with less IL-4 and IL-13 production than extrinsic AD (Akdis and others 1999a). Furthermore, IL-10 appears to play an important immunoregulatory role in both forms of AD (Laouini and others 2003; Howell and others 2005).

Canine atopic dermatitis mediated by allergen-specific IgE antibody is measured by demonstrable intradermal tests or *in vitro* tests. On rare occasions, however, reactivity to allergen is not demonstrable in patients that otherwise appear to be suffering from classical AD. Moreover, many normal dogs show positive intradermal skin tests to environmental allergens or have elevated allergen-specific IgE, and yet show no disease.

The pathogenic role played by allergen-specific IgE in atopic dermatitis is supported by numerous studies but the absolute requirement for IgE has been questioned. Other components of the immune system, i.e. Langerhans cells, T cells, and eosinophils, as well as changes in the inflammatory milieu with chronicity, are also actively involved as the disease progresses.

A summary of the current thoughts as to the possible pathogenesis of atopic dermatitis might go as follows: inhaled or percutaneously absorbed allergens encounter allergen-specific IgE on Langerhans' cells, whereupon the allergens are trapped, processed, and presented to allergen-specific T lymphocytes. There is a subsequent preferential expansion of allergen-specific Th2 cells, which produce IL-3, IL-4, IL-5, IL-6, IL-10 and IL-13. The imbalance in allergen-specific Th2 cells and

allergen-specific Th1 cells culminates in enhanced production of allergen-specific IgE by B lymphocytes. With chronicity, changes occur in cytokine expression with lower levels of IL-13 and increased IFN-γ.

1.1.2 The prevalence of atopic dermatitis in human and dogs

The prevalence of AD in US children is 17.2% (Laughter and others 2000) and is similar to the 15.6% prevalence described in the European childhood population (Schultz Larsen and others 1996) and the 24% prevalence in 5- to 6-year-old children in Japan (Sugiura and others 1998). The manifestation of AD in such a sizable proportion of the paediatric population in affluent western societies represents a marked increase over the past several decades. The main reason for this increase may relate to our modern environment because the gene pool has not changed substantially over this relatively short period of time. It has been shown that AD occurs more frequently in urban areas, in smaller families and in higher socioeconomic classes, suggesting that exposure to antigenic pollutants and lack of exposure to infectious agents or other antigenic triggers (particularly those that favour a Th1 type T-cell response) early in life might play a role in the development of the dermatitis (von Mutius 1999; von Mutius 2000).

The prevalence of AD in the canine population has not been studied using reliable epidemiological data. In an early report, the prevalence of canine atopic dermatitis (CAD) was estimated to be 15% (Chamberlain 1974) and recently, estimates of 3-15% (Reedy and others 1997) and around 10% (Scott and others 2001) have been stated. However, the true prevalence of AD in the general dog population remains unknown.

It is speculated that the prevalence of AD has increased in dogs as in humans because dogs are exposed to the same environmental influences as humans. Furthermore, over the last few decades dogs are spending more time indoors, thus exposure to common indoor allergens such as house dust mites has also increased. Early vaccination and the practice of parasitic control and usage of antibiotics is more

common (Hillier and Griffin 2001b). Robust and valid epidemiological data is required to support the above theory.

1.2 Genetic and predisposing factors in atopic dermatitis

1.2.1 Human atopic dermatitis

A genetic background for AD has long been proposed because the disease tends to cluster in families (Kaiser 2004). However, unravelling the genetic basis of AD is challenging because it is a multifactorial disease arising from a complex interaction of several genetic and environmental factors. The concordance rates for monozygotic twins have been estimated between 72 and 86%, compared to 21-23% for dizygotic twins (Schultz Larsen and Holm 1985; Larsen and others 1986; Schultz Larsen 1993). The magnitude of the concordance rates indicates that genetic factors are decisive in the development of AD, whereas incomplete concordance for AD among monozygotic twins suggests elusive non-genetic factors.

In another report, Uehara and Kimura (Uehara and Kimura 1993) studied 270 adults with AD and found that 60% of their offspring were also affected; the prevalence of the skin condition was 81% when both parents were affected by AD, 59% when only one parent was affected by AD and the other one had respiratory atopy, and 56% when only one of the parents was affected by AD. This study demonstrated the presence of an autosomal dominant inheritance pattern of AD.

To identify candidate regions encoding genes for AD, genome-wide screens followed by positional cloning are employed. Results of various studies show no substantial overlap and careful interpretation is necessary. Regions on chromosomes 3q, 3p, 17q and 18q showed suggestive evidence for linkage in at least two genome-wide screening studies, indicating promising candidate regions for AD (Lee and others 2000; Cookson and others 2001; Bradley and others 2002; Haagerup and others 2004). Interestingly, the susceptibility regions identified for AD show little overlap with asthma susceptibility regions, suggesting that separate genes might be involved in the pathogenesis of the different atopic disorders (Hoffjan and Epplen 2005).

Instead, some of the identified regions overlap with susceptibility regions for psoriasis, another chronic skin disease (Cookson and others 2001). Thus, genes expressed in the skin might play an important role in AD pathogenesis, in addition to genes influencing the atopic diathesis.

Following a genome-wide screen, positional cloning techniques are used to identify the gene(s) influencing disease susceptibility in the linked region. Although no AD gene has been identified by positional cloning yet, it is likely it will be discovered by this approach in the near future.

Candidate gene association study is also useful in identifying susceptibility genes for AD. Variations in known genes whose biological functions implicate them in the pathophysiology are compared between unrelated cases and controls. Most candidate gene association studies focused on genes thought to play a role in allergic disorders in general, e.g. genes in the cytokine gene cluster region on chromosome 5q31. Variation in three genes has been associated with AD in at least four different studies; the interleukin-13 (*IL13*) gene (Liu and others 2000; Tsunemi and others 2002; He and others 2003; Hummelshoj and others 2003), interleukin 4 receptor alpha (*IL4RA*) gene (Hershey and others 1997; Oiso and others 2000; Tanaka and others 2001; Callard and others 2002; Novak and others 2002; Hosomi and others 2004) and serine protease inhibitor Kazal-type 5 (*SPINK5*) gene (Walley and others 2001; Kato and others 2003; Nishio and others 2003; Kabesch and others 2004; Kusunoki and others 2005). However, whether *IL13*, *IL4RA* or *SPINK5* genes represent atopy susceptibility loci remains to be unravelled.

1.2.2 Canine atopic dermatitis

Distinct breed predispositions (e.g. West Highland white terrier, Labrador retriever, Boxer), familial involvement and limited breeding trials have demonstrated that canine AD is genetically programmed (Schwartzman and others 1983; Halliwell and Gorman 1989; Reedy and others 1997; de Weck and others 1997; DeBoer and Hill 1999). A pilot study showed no significant difference in any single gene frequency

of the different Dog Leukocyte Antigen (DL-A) groups between normal and atopic dogs (Vriesendorp and others 1975), but the combination of haplotypes DL-A3 and R15 was found significantly more often in atopic dogs and might be associated with an increased susceptibility to AD (Vriesendorp and others 1975). However, in one study only 13 out of 72 dogs of the progeny of patients with AD developed clinical signs of AD (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 exposure to allergens (de Weck and others 1997), which emphasises the importance of both environmental and genetic factors in the development of the production of IgE. These findings suggest that, similar to humans, canine AD is the end result of a complex interaction between genetic traits and environmental influences. However, studies of genetic factors in dogs are limited and need further investigation.

1.3 Clinical manifestations of atopic dermatitis

1.3.1 Human atopic dermatitis

Atopic dermatitis in humans is a common chronic inflammatory skin disease often proceeding the development of asthma and allergic disorders, such as food allergy, allergic rhinoconjunctivitis and asthma (Spergel and Paller 2003; Leung and others 2004). It is one of the most common skin disorders seen in infants and children and has its onset before 5 years of age in at least 85% of affected individuals (Kay and others 1994). The clinical course often results in spontaneous complete remission at puberty or shortly after puberty in 40% to 60% of patients, although patients who do not show clearance might show improvement with advancing age (Williams and Strachan 1998; Wuthrich 1999).

Clinical manifestations include pruritus and chronic or relapsing eczematous lesions that are typically localised to the ante-cubital and popliteal flexural areas in patients aged 2 years or older whereas infantile AD generally involves the scalp, face, cheeks, and extensor surfaces of the extremities (Leung and Bieber 2003). The acute lesions of AD present as pruritic, erythematous, excoriated papules with extensive serous

exudate superimposed on a background of erythema (Leung and others 1998). Repeated excoriations and trauma lead to the chronic lesions of AD, which are characterised by thickened plaques with increased markings (lichenification) and dry, fibrotic papules (Leung and others 1998). Provocative factors can be psychological, contact irritants, hormonal effects, heat, humidity and perspiration (Rothe and Grant-Kels 1996; Friedmann 1999) and colonisation of lesional skin with staphylococci and *Malassezia* yeasts is commonly observed (Halbert and others 1995; Herz and others 1998).

Affected individuals of AD must cope with a significant psychosocial burden, in addition to the medical aspects of the disease. The discomfort of intense pruritus often leads to sleep disturbance, reduced functional capacity, and impaired daily activity performance. Because AD is primarily a disease of childhood, the quality of life for both the patient and family members, especially parents, is also affected (Spergel and Paller 2003).

1.3.2 Canine atopic dermatitis

The typical age of onset of canine AD is reported to be between 6 months and 3 years (Griffin and DeBoer 2001a). Initial clinical signs of AD can be seasonal or nonseasonal, depending upon the allergens involved, but the majority of dogs with AD eventually exhibit nonseasonal clinical signs (Scott and others 2001). In dogs, pruritus has been considered a hallmark of AD and is typically manifested by foot licking, scratching, and nose or head rubbing. Clinical manifestations include pruritus of the face, ears, paws, distal extremities, anterior elbows, and ventrum, or some combination thereof. Primary lesion, such as erythema, in pruritic areas is noted in some dogs, while in others no visible primary lesion is presented. Secondary lesions in atopic dogs are usually those associated with chronic pruritus, self-trauma, secondary bacterial pyoderma, secondary *Malassezia* dermatitis, secondary seborrhoeic skin disease or microbial overgrowth. These above-mentioned conditions may result in complete or partial alopecia, salivary staining, papules, pustules, circular crusted papules, excoriations, dry lusterless hair, scaling,

hyperpigmentation and lichenification (Scott and others 2001). Although non-cutaneous signs are uncommon in atopic dogs, conjunctivitis, reverse sneezing, rhinitis, and asthma-like symptoms may occur.

1.4 Diagnosis and management

1.4.1 Diagnosis

1.4.1.1 Fundamental concepts in clinical diagnosis

The clinical signs of AD are variable, and there is no pathognomonic clinical feature that, if present, indicates the presence of AD. Initial diagnosis of AD is based upon the fulfilment of at least a part of a constellation of strongly associated clinical criteria along with careful and complete elimination of other relevant differential diagnoses and concurrent problems (DeBoer and Hillier 2001a). A list of diagnostic criteria developed in humans has been extrapolated and modified for use in canine AD (Table 1.1) (Willemse 1986; Prelaud and others 1998).

Following clinical diagnosis, laboratory or clinical evaluations such as serum-based or intradermal skin tests are conducted to provide additional evidence to substantiate the diagnosis and also to identify offending allergens. Results of allergy tests provide information for selection of candidate allergens for allergen-specific immunotherapy and also as a basis for institution of allergen avoidance measures.

Table 1.1 Clinical diagnostic criteria of canine atopic dermatitis

Willemse (1986)	Prelaud et al (1998)
Major features	*Major criteria*
Patients must have at least three of the following features: · Pruritus · Typical morphology and distribution: Face and/or digital involvement or lichenification of the flexor surface of the tarsal joint and/or the extensor surface of the carpal joint · Chronic or chronically-relapsing dermatitis · Individual or family history of atopy, and/or breed predisposition	Patient must have at least three out of the following five features: · Corticosteroid-sensitive pruritus · Bilateral cranial erythematous pododermatitis · Erythema of pinnae · Cheilitis · Appearance of first signs between the ages of 6 months and 3 years
Minor features	
At least three of the following features also should be present: · Onset of symptoms before 3 years · Facial erythema and cheilitis · Bacterial conjunctivitis · Superficial staphylococcal pyoderma · Hyperhidrosis · Immediate positive intradermal test to inhalants · Elevated serum allergen-specific IgE · Elevated serum allergen-specific IgGd	

1.4.1.2 Serum-based allergy tests

Allergen-specific IgE assays detect IgE antibody directed specifically against a panel of allergens thought to be clinically relevant to the patient's disease. The radioallergosorbent test (RAST), enzyme-linked immunosorbent assay (ELISA) and liquid-phase immunoenzymatic assays are commercially used to detect allergen-specific IgE in the serum. The fundamental principle of these assays is identical: the patient serum is first reacted with an individual allergen extract bound to either a solid support or in liquid phase. Unreacted antibodies are washed away, and the allergen-bound IgE is detected using a reagent specific for IgE previously coupled to an enzyme or reporter substrate. The amount of bound IgE-specific reagent is quantified appropriately. Allergy tests are usually carried out by commercial veterinary allergy laboratories; unfortunately great variation exists due to factors including source of allergen extracts, reacting phase of allergen, nature of IgE-specific detection reagent, signal molecule, sample pre-processing, reporting and interpretation of results, as well as standardisation and quality control procedures (DeBoer and Hillier 2001b). Allergen-specific IgE serological tests are never completely sensitive, nor completely specific; therefore, careful interpretation of the results is warranted.

Allergen-specific IgE serological tests should only be considered if there is strong clinical evidence for atopic dermatitis, and after all other differential diagnoses have been ruled out. They are merely tools to aid diagnosis and therapy, and not definitive diagnostic tests.

1.4.1.3 Intradermal test (IDT)

The primary utility of intradermal testing is in the demonstration of IgE-mediated hypersensitivity to various environmental allergens based on skin reactivity. It is based upon the release of histamine from mast cells upon interaction of surface-bound IgE with intradermally-injected allergenic extracts. Immediate skin test reactions are read 10-20 minutes after injection with the aid of incident light.

Reactions are subjectively graded with reference to positive and negative controls based on the degree of erythema and diameter, height and turgidity of the wheal.

There is a real need for determination of the major allergens in dogs, standardisation of allergen extracts, standardisation of IDT technique, and selection of the best criteria for the interpretation of immediate- and late-phase skin reactions in the dog (Hillier and DeBoer 2001a).

Intradermal testing is only indicated in animals with a history and clinical signs consistent with atopic dermatitis and should only be performed after other pruritic diseases have been ruled out.

1.4.2 Management

1.4.2.1 General principles of therapy

Permanent curative therapy for canine atopic dermatitis is currently unavailable. Treatment is usually required for life and therapeutic modifications over the life of the dog are necessary. The management of canine AD is multifactorial and includes various combinations of allergen avoidance, topical therapies, allergen-specific immunotherapy, fatty acids and antihistamines, antimicrobial drugs, anti-inflammatory drugs, systemic glucocorticoids, and occasionally immunosuppressive drugs (Olivry and Sousa 2001; Scott and others 2001). Education of the client is also of key importance to the successful long-term control of the atopic dog. A treatment plan should be developed for each individual taking account of multiple variables, including seasonality, severity of skin lesions, cost, willingness and ability of the client to administer the treatment, and acceptability and risk to the patient. In general, over 90% of atopic dogs can be satisfactorily controlled (Scott and others 2001).

1.4.2.2 Glucocorticoids

Glucocorticoids are among the most commonly used anti-inflammatory and immunosuppressive drugs used in veterinary practice. Their mechanism of action

arises from transactivation (glucocorticoid response element (GRE) binding) or transrepression (interaction with transcription factors) (Barnes 1998). The main anti-inflammatory effects of glucocorticoids is due to repression of gene activation by interference with multiple ubiquitous transcription factors (de Bosscher and others 2000). Glucocorticoids also activate anti-inflammatory genes to translate proteins such as lipocortin-1, which is thought to cause a reduction in the action of phospholipase A2 on cell membranes, which results in inhibition of the arachidonic acid cascade (Croxtall and others 1996).

When used to treat canine AD, glucocorticoids should be used at as low a dose as possible, as infrequently as possible and in alternate day regimes whenever possible to reduce potential side effects.

1.4.2.3 Cyclosporine

Cyclosporine (cyclosporin A, CsA) is a cyclic polypeptide isolated from the *Tolypocladium inflatum* fungus that possesses immuno-modulating properties from its capability to block the activity of cytoplasmic calcineurin phosphatase, thereby preventing the induction of genes encoding cytokines and their receptors (Mascarell and Truffa-Bachi 2003). It exhibits anti-allergic properties because it inhibits (1) the functions of cells that initiate immune reactions (i.e. Langerhans cells and lymphocytes) and (2) the functions of effector cells of the allergic response (i.e. mast cells and eosinophils) (Marsella and Olivry 2001). It has recently been shown that the administration of CsA for the treatment of canine AD is as effective as that of glucocorticoids, and adverse effects are minimal (Steffan and others 2006).

1.4.2.4 Antihistamines

The beneficial effect of antihistamines for control of pruritus in dogs has been established (Paradis and others 1991a; Paradis and others 1991b; Miller Jr. and others 1993) although the true efficacy of antihistamines to relieve the pruritus of canine AD has not been confirmed. Sedative actions of first generation H1-blockers

may in part be responsible for clinical benefit. The effects of antihistamines may be additive or synergistic when used along with other medications such as essential fatty acid (EFA) supplements or corticosteroids (Paradis and others 1991b; Scott and Miller 1993a; Scott and Miller 1993b).

1.4.2.5 Essential fatty acid supplements

Essential fatty acids (EFAs) possess multiple anti-inflammatory and immuno-modulating properties and it has been shown that EFAs are beneficial in the management of pruritus in dogs (Bond and Lloyd 1992; Scarff and Lloyd 1992; Scott and Miller 1993b; Bond and Lloyd 1994; Sture and Lloyd 1995b). Their mechanisms of action in the treatment of allergic inflammation includes the modulation of prostaglandins (PG) and leukotrienes (LT) (Bjorneboe and others 1987; Lee and others 1991; Broughton and others 1997), the inhibition of cellular activation and cytokine secretion (Endres and others 1989; Carrick and others 1994; Rossetti and others 1995; de Caterina and Libby 1996) as well as the alteration of the composition and function of the epidermal lipid barrier (Linde 1992; Fartasch and Diepgen 1992; Marsh and others 2000). The synergistic effect of EFAs and antihistamines (Paradis and others 1991a; Scott and Miller 1993b; Paterson 1995) or glucocorticoids (Scott and Miller 1993a; Scott and Miller 1993b; Bond and Lloyd 1994) has also been described. Taken together, because of the low potential for risk and their good potential for benefit, trial therapy with optimised diets or supplements is often warranted as part of the overall management of dogs with AD.

1.4.2.6 Allergen-specific Immunotherapy

Allergen-specific immunotherapy is a valuable therapeutic option for non-seasonal atopic dermatitis (Scott and others 2001). ASIT is the only form of therapy that specifically addresses the underlying allergic reaction, as compared to drugs that counteract the effects of the allergic reaction. It is defined as the practice of administering gradually increasing quantities of an allergen extract to an allergic subject to ameliorate the symptoms associated with subsequent exposure to the

causative allergen (Bousquet and others 1998). Possible mechanisms underlying the mode of action of ASIT are discussed in detail in section 1.9.2.

1.5 Cutaneous micro-organisms and atopic dermatitis

Patients with atopic dermatitis are highly susceptible to certain cutaneous bacterial, fungal and viral infections (Lubbe 2003), and cutaneous infections are indeed frequently observed in dogs and humans with AD. Skin infections could be a consequence of changes in the skin caused by AD itself, or it could be an important component of the pathogenesis of AD through their effects on the immune system and by perpetuating the cutaneous inflammatory response (DeBoer and Marsella 2001c).

1.5.1 Staphylococci and superantigens

Bacterial skin disease is a very common and important complication. The major microbial pathogens involved are the pathogenic staphylococci; *Staphylococcus aureus* in man and *S. intermedius* in the dog. About 80% of human patients with AD are colonised with *S. aureus* (David and Cambridge 1986; Hoeger and others 1992; Monti and others 1996), and numbers of staphylococci are elevated both in lesional and, to a lesser extent, non-lesional skin of atopic dermatitis patients (Leyden and others 1974; Lubbe 2003). Furthermore, the degree of colonisation correlates with the severity of clinical signs in human atopic dermatitis (Herz and others 1998). *S intermedius* is present in muco-cutaneous sites, skin surface and hair coat of healthy dogs, but the reason why atopic dogs exhibit increased susceptibility to staphylococcal pyoderma is not fully understood and is probably multifactorial.

Adherence by bacteria to host cell surfaces is recognised to be an important virulence factor and is appreciated to be an initial step in the colonisation and subsequent invasion (Beachey 1981; Cree and Noble 1995). Increased adherence by *S. aureus* in humans and by *S. intermedius* in dogs to corneocytes have been demonstrated and is one possible explanation for the high incidence of staphylococcal infection in atopic

patients (Cole and Silverberg 1986; Simou and others 2005; McEwan and others 2005). Furthermore, there is a causative relationship between the numbers of bacteria present on the skin and the severity of disease in AD patients, whilst treatment-induced removal of the bacteria is associated with improvement in skin lesions in most cases (Nilsson and others 1992; Guzik and others 2005; Baker 2006). Other factors possibly involved in the altered skin colonisation by *S. aureus* in AD are altered epidermal barrier, defective bacterial clearance, and decreased innate immune responses (Baker 2006).

Exacerbation of the inflammatory immune response caused by increased colonisation of skin by *S. aureus* is largely mediated by the release of staphylococcal enterotoxins (SE), such as SEA, SEB and toxic shock syndrome toxin (TSST)-1, also referred to as superantigens (Proft and Fraser 2003). Staphylococcal superantigens activate T cells bearing superantigen-specific TCR Vβ families by directly linking MHC II and TCR molecules in a non-antigen specific manner (Huston 1997; Baker 2006). Superantigens are capable of activating a large number of T cells (up to 30% of all T cells) without prior antigen processing and are different from regular antigens, which need processing by APCs and activate only specific T cells (usually less than 0.1% of all T cells) (Saloga and Knop 1999; Sugimoto and others 2006). In humans, there is an association between these Vβ domains and house dust mite specific TCRs (Herz and others 1998).

In addition to T cells, superantigens can also mediate effects on other cell types and are strongly pro-inflammatory. Superantigens modulate the effector function of eosinophils by inhibiting apoptosis, increasing expression of activation antigens on the eosinophil surface, and enhancing the oxidative burst of eosinophils *in vitro* (Wedi and others 2002). They also stimulate Langerhans cells and macrophages to produce IL-1, TNF-α and/or IL-12 (Leung 2000), which up-regulate the expression of adhesion molecules on endothelial cells (ICAM-1 and VCAM-1) or increase CLA expression on T cells, thus facilitating the recruitment of CLA+ memory T cells to the skin (Baker 2006). TSST-1 induces MHC class II and co-stimulatory molecules on B cells, augmenting IgE production (Hofer and others 1995).

SEA, SEB, SEC, SED, SEE 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 can also act as conventional antigens. The majority of patients with AD mount an IgE response to staphylococcal superantigens and this has been linked to exacerbation AD by activating mast cells, basophils and other Fcε-receptor bearing cells armed with the relevant antitoxin (Leung and others 1993). Anti-staphylococcal IgE has been detected in dogs with AD complicated by recurrent staphylococcal pyoderma (Morales and others 1994), but its significance is unclear.

1.5.2 *Malassezia* and atopic dermatitis

Malassezia is part of the normal skin flora but a proportion of patients with AD develop a hypersensitivity response to cutaneous *Malassezia* (Nordvall and Johansson 1990; Broberg and others 1992). Most healthy individuals develop IgG antibodies to *Malassezia*, but in 30-80% of AD patients, IgE and/or T cell reactivity to the organism is present (Scheynius and others 2002). Production of IgE antibodies against *Malassezia* occurs very frequently in AD, and rarely in healthy individuals or non-atopic patients with *Malassezia* dermatoses (Wessels and others 1991). Histamine release tests have confirmed the biological activity of circulating *Malassezia*-specific IgE antibodies in 70% of AD patients, supporting a role for these antibodies in the disease process (Nissen and others 1998). PBMC proliferative response to *Malassezia* is also significantly higher in AD patients than in healthy individuals (Tengvall Linder and others 1996). *Malassezia* is capable of modulating cytokine production (favouring a Th2-type response) and can play a role in maintaining IgE-mediated skin inflammation in AD (Tengvall Linder and others 1996). However, no evidence has been found for superantigenic activity by *Malassezia* in patients with AD (Johansson and others 1999).

Malassezia dermatitis and otitis are common complications in dogs suffering from AD. Intradermal testing using *Malassezia* extracts demonstrated that dogs with AD and *Malassezia* dermatitis exhibit significantly greater responses than those with AD and no *Malassezia* dermatitis (Morris and others 1998). Specific IgE antibody levels to *M. pachydermatis* are significantly higher in atopic dogs than in healthy dogs, and are not dependent on the presence of elevated cutaneous numbers of *Malassezia* (Nuttall and Halliwell 2001a; Chen and others 2002b). However, PBMCs isolated from atopic and healthy dogs have similar responses to Malassezia extracts *in vitro* (Morris and others 2002). Whether *Malassezia* species are actively involved in the pathogenesis of atopic dermatitis remain to be established.

1.6 Atopic dermatitis and house dust mite allergens

1.6.1 Mites found in household dust (domestic mites)

Traditionally, the common name 'house dust mite' has been used collectively to include those members of the family Pyroglyphidae (order Acari, suborder Astigmata) that live permanently in house dust. However, mites belonging to other families are also present in house dust, and the term 'domestic mites' is proposed to be used for the various free-living mites that inhabit human dwellings, including house dust mite (family Pyroglyphidae), storage mites (families Acaridae, Glycyphagidae and Chortoglyphidae) and their predator mites (family Cheyletidae) (Platts-Mills and others 1997).

Most of the 46 species of Pyroglyphidae are nest-dwellers or feather-associates of birds (Wharton 1976), and only 13 are recorded from house dust, of which six, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Hirstia domicola*, *Malayoglyphus intermedius*, *Malayophyghus maynei*, and *Euroglyphus maynei* have been recorded in temperate human habitats repeatedly and throughout the world. Another two species have more limited distributions, *Dermatophagoides siboney*, so far restricted to Cuba, and *Dermatophagoides microceras* (frequently misidentified as *Dermatophagoides farinae*), predominantly found within Europe. In tropical climates, the storage mite *Blomia tropicalis* (family Glycyphagidae) can be a

prevalent mite in dwellings (Fernandez-Caldas and others 1993), along with other Pyroglyphid mites.

1.6.2 Increasing exposure to house dust mites

The increased prevalence of AD seen over the past several decades may relate to increasing exposure to dust mite allergens due to changes in Western lifestyles and housing conditions. Mite growth and proliferation are dependent on several factors, particularly temperature and humidity. Features of modern housing such as insulation, central heating and reduced ventilation have raised indoor temperature and humidity, which significantly increases the level of mite infestation in homes (Munir and others 1995; Hirsch and others 2000).

1.6.3 The importance of *Dermatophagoides* species house dust mites

The most important allergy-causing mites found in homes worldwide are the house dust mites *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, *Euroglyphus maynei*, and the storage mite *Blomia tropicalis* (Arlian and others 2002). It has been shown that most homes contain multiple mite species, and the most prevalent mite species and allergens in homes differ geographically, between homes within a geographical region, and among areas within a home (Arlian and others 1992). Information regarding the mite species present in a geographical area is crucial when performing diagnostic testing and prescribing immunotherapy.

The name *Dermatophagoides* is derived from the Greek words dermis meaning 'skin', phagos, referring to feeding, and the suffix –oides meaning 'to look like', and it roughly translates as 'thing that looks like those that eat skin.' *D. pteronyssinus* prefers more humid conditions, and *D. farinae* is most common in dry continental climates. Data from intradermal and serological testing suggest that *D. farinae* is the most common antigen implicated in canine atopic dermatitis in the UK (Sture and others 1995a; Nuttall and others 2001b; Foster and others 2003), Europe (Noli and others 1996; Bensignor and Carlotti 2002), the USA (Hillier and others 2000; Zur

and others 2002a), Australia (Mueller and others 2000) and Japan (Yamashita and others 2002). However, studies investigating house dust mite species found in British homes rarely identify the presence of *D. farinae* (Eaton and others 1985; Colloff 1987; Young and others 1990; Hart and Whitehead 1990; Chen and others 2002b; Jackson and others 2005; Raffan and others 2005; Nuttall and others 2006). The high proportion of positive intradermal and serological reactions to *D. farinae* in CAD from geographical areas where prevalence and exposure is very low requires further work to clarify whether this reflects true hypersensitivity to *D. farinae* or whether cross-reactivity to other allergens is occurring (Jackson and others 2005).

1.6.4 *Dermatophagoides* species allergens

1.6.4.1 Allergens relevant to humans

Allergens from the house dust mites *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* are major environmental trigger factors for atopic dermatitis in human (Thomas and Smith 1999). More than 17 different *Dermatophagoides* allergens have been identified (Table 1.2). The group 1 (cysteine proteases) and 2 allergens are recognised to be of primary importance because of their high IgE-binding frequency (Chua and others 1988; Dilworth and others 1991) and can account for much of the allergenicity of extracts. The groups 3 (trypsin), 6 (chymotrypsin) and 9 (collagenase) family members are serine proteases which provoke hyper-reactivity as well as tissue inflammatory reactions (Yasueda and others 1993; Smith and others 1994; Nishiyama and others 1995; King and others 1996; Bennett and Thomas 1996; Kawamoto and others 1999). The groups 4 (amylase), 5, 7, 8 (glutathione S-transferase (GST)), and 10 (tropomyosin) members have been identified as additional major allergens (Lake and others 1991; O'Neill and others 1994; Lin and others 1994; Shen and others 1995; Aki and others 1995; Mills and others 1999). Recent studies on high-molecular size allergens have elucidated new major antigens including the groups 11 (paramyosin, 98 kDa), 14 (apolipophorin-like 177 kDa molecule) and 15 (chitinase-like protein, identified using canine IgE) (Fujikawa and others 1998; Tsai and others 1999; Epton and others 1999; McCall and others 2001).

Although many proteins produced by *Dermatophagoides* mites induce IgE antibody, the majority of the allergenic activity resides in a small number of dominant allergens. Frequently, over 50% of the IgE binding activity of mite extracts could be attributed to the group 1 and 2 allergens (Chapman and others 1980; van der Zee and others 1988). However, recent studies suggest that 13 allergens of *Dermatophagoides* mites elicit IgE response in more than 40% of mite allergic humans (Thomas and Smith 1999). Further research is required to clarify the importance of individual allergens.

Table 1.2 Allergens identified in *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*

Allergen Group	Allergen Name	Function	MW (kDa)	Reference and/or accession number
Group 1	Der p 1	cysteine protease	25	(Chua and others 1988)
	Der f 1			(Dilworth and others 1991)
Group 2	Der p 2	epithelial secretion	14	(Chua and others 1990; Smith and others 2001a; Smith and others 2001b)
	Der f 2			(Trudinger and others 1991; Nishiyama and others 1993)
Group 3	Der p 3	trypsin	30	(Smith and Thomas 1996)
	Der f 3			(Smith and Thomas 1996)
Group 4	Der p 4	amylase	60	(Lake and others 1991)
Group 5	Der p 5	unknown	14	(Tovey and others 1989)
Group 6	Der p 6	chymotrypsin	25	
Group 7	Der p 7	unknown	22/28	(Shen and others 1993)
	Der f 7		24-31	(Shen and others 1995); Q26456
Group 8	Der p 8	glutathione S-transferase		(O'Neill and others 1994)
Group 9	Der p 9	collagenolytic serine protease	30	(King and others 1996)
Group 10	Der p 10	tropomyosin	37	Y14906
	Der f 10			(Aki and others 1995)
Group 11	Der p 11	paramyosin	103	(Lee and others 2004); AY189697
	Der f 11		98	(Tsai and others 1999)
Group 14	Der p 14	apolipophorin like protease	177	(Epton and others 1999; Epton and others 2001)
	Mag 3			(Fujikawa and others 1996; Kawamoto and others 2000)
Group 15	Der p 15	chitinase	59/61	(O'Neil and others 2006)
	Der f 15		98/105	(McCall and others 2001); AF178772
Group 16	Der f 16	gelsolin/villin	53	(Kawamoto and others 2002b)
Group 17	Der f 17	EF calcium-binding protein	53	(Kawamoto and others 2002a)
Group 18	Der p 18	chitinase	49	(O'Neil and others 2006)
	Der f 18		60	(Weber and others 2003)
Group 20	Der p 20	arginine kinase	40	Thomas unpublished data
Group 21	Der p 21		14	DQ354124

1.6.4.2 Allergens relevant to dogs

Dermatophagoides species are equally important in causing atopic dermatitis in dogs (Sture and others 1995a). The spectrum of *Dermatophagoides* allergens relevant to dogs is less clear, but the low molecular weight molecules defined as major allergens for humans do not appear to be major allergens for dogs. Studies using anti-canine IgGd (Noli and others 1996), anti-canine IgE (Nuttall and others 2001b) and recombinant FcεRIα (Shaw S.C. 2000; McCall and others 2001) 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 f 1 using FcεRIα in one study (McCall and others 2001), others detected very little binding to group 1 and 2 allergens using either FcεRIα (Shaw S.C. 2000), anti-canine IgE (Nuttall and others 2001b) or anti-canine IgGd (Noli and others 1996). Another study using anti-canine IgE reported that 45 per cent of atopic sera recognised both Der p 1 and 2 and Der f 1 and 2 (Masuda and others 1999). These conflicting results could arise from the variable purity of the allergen preparations (Le Mao and others 1998) and specificity and sensitivity of different antisera (Steward and Male 1998).

The major *Dermatophagoides* allergen in canine AD has recently been cloned, expressed and named Der f 15 (McCall and others 2001). The majority of atopic dog sera also recognised 98/109 kDa allergens in *D. pteronyssinus* (Shaw S.C. 2000). Another 60-kDa major *Dermatophagoides* protein (Der f 18) (Weber and others 2003) is also important in canine AD and is recognised in 57 % to 77 % of dust mite-sensitised dogs. Both Der f 15 and Der f 18 are homologous to insect chitinases and are located in the mite gut suggesting it has a function in the digestion rather than in moulting (McCall and others 2001; Weber and others 2003). The importance of other mite allergens in the involvement of canine AD remains the subject of intensive study.

1.7 The role of B cells and immunoglobulins in atopic dermatitis

The typical sequence of events in atopic dermatitis consists of exposure to an antigen, activation of T cells specific for the antigen, production of IgE antibody, binding of the antibody to Fc receptors of mast cells, and triggering of the mast cells by re-exposure to the antigen, resulting in the release of mediators from the mast cells and the subsequent pathologic reaction (Abbas and Lichtman 2003). The pathophysiology involves a complex series of interactions between resident and infiltrating cells orchestrated by pro-inflammatory cytokines and chemokines (Boguniewicz and Leung 2006).

1.7.1 IgE

IgE antibody is responsible for sensitising mast cells and provides recognition of antigen in allergy. FcεRI is an IgE receptor expressed mainly on mast cells and basophils, but it is also found on Langerhans cells. The affinity of this receptor for IgE is extremely high (Kd > 10^{-10} M), and therefore it readily binds monomeric IgE molecules even at the low normal plasma concentrations of this antibody isotype. Another IgE receptor with lower affinity (Kd > 10^{-7} M) termed FcεRII / CD23 has been found on human B lymphocytes, monocytes and eosinophils but its role in atopic dermatitis is less clear.

The high-affinity IgE receptor expression facilitates ingestion of allergens by Langerhans cells via FcεRI-mediated uptake. It has been shown that Langerhans cells in the lesional skin of human patients with atopic dermatitis display up regulated expression of FcεRI (Stingl and Maurer 1997; Kraft and others 1998) and that Langerhans cells from AD skin lesions are 1000-fold more efficient in presenting house dust allergen to T cells than are Langerhans cells that lack surface IgE (Maurer and others 1995).

In addition to assisting allergen presentation, IgE antibody also plays a pivotal role at the effector level through the binding to FcεRI on mast cells, basophils and

eosinophils. Upon re-exposure to offending allergen, IgE cross-linking of $Fc\varepsilon RI^+$ cells by specific antigens results in the release of variety of preformed (e.g. histamine, etc.) and *de novo* synthesized chemical mediators (e.g. peptide leukotrienes, prostaglandins, etc.) (Marone and others 1986; Marone and others 1987), and cytokines (Brunner and others 1993; Li and others 1996) that exert their effects by interacting with specific receptors on target organs (Vigorito and others 1986; Marone and others 1988). Cross-linking of surface IgE by allergen, furthermore, induces FcεRI and IgE synthesis (Helm and others 1998).

Serum IgE concentrations increase from the age of 1 year to 4 years and then plateau, which coincides with the peak age of onset of atopic dermatitis in dogs (Racine and others 1999). Female dogs have significantly higher mean IgE concentrations than do male dogs (Racine and others 1999), while in humans higher mean IgE values are observed in males than in females (Warren and others 1982; Omenaas and others 1994). However, no consistent female bias in atopic dermatitis has been found (Scott and others 2001).

1.7.1.1 Total serum IgE in atopic dermatitis

Total serum IgE concentrations are elevated in 43% to 82% of human patients with AD (Juhlin and others 1969; Johnson and others 1974). Although serum IgE has a short half life of 5 to 7 days, the levels do not fluctuate in close association with clinical flares and remissions (Johansson and others 1970). When severe AD is treated with systemic steroids, the clinical improvement that ensues is not accompanied by a decrease in the serum level of IgE. The IgE level returns to normal when patients with a history of severe dermatitis have been free from their disease for at least 2 years (Johansson and others 1970).

In dogs however, although serum IgE concentrations are considerably greater than those in humans (Hammerberg and others 1997), there is no significant difference between dogs with AD and clinically normal controls (Wilkie and others 1990; Hill and others 1995; Jackson and others 1996; Fraser and others 2003) and measurement

of total serum IgE would be of no benefit in the preliminary clinical investigation of a suspected atopic dog (Hill and others 1995). Various authors have speculated as to the reason for these differences between dog and humans and it is currently assumed that greater exposure of the canine population to parasites accounts for this discrepancy.

1.7.1.2 Allergen-specific IgE in atopic dermatitis

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). Serological or skin tests detecting allergen-specific IgE are routinely used to identify allergens for avoidance or specific immunotherapy (Scott and others 2001). However, the absolute requirement for IgE is questioned. Two forms of AD have been delineated in humans, including an extrinsic form associated with IgE-mediated sensitisation involving 70% to 80% of patients and an intrinsic form without IgE-mediated sensitisation involving 20% to 30% of the human patients (Novak and others 2003b). Allergen-specific IgE is also present in healthy humans and dogs and the titres do not fluctuate consistently during exacerbations, remissions or treatment, suggesting that heterogeneous IgE antibodies with variable receptor binding affinities might exist (Halliwell and others 1998; Marone and others 1999; Griot-Wenk and others 1999). The presence of positive skin tests in some healthy individuals though (Halliwell and others 1998; Lian and Halliwell 1998), suggests that other tolerogenic mechanisms arc also present.

1.7.1.3 House dust mite specific IgE in atopic dermatitis

The biochemical and IgE binding characteristics of the known house dust mite allergens in humans are summarised in Table 1.3 (Thomas and others 2002). There is extensive structural and immunochemical data on the group 1 and 2 allergens, and more than 80% of mite-allergic patients have IgE antibodies to these proteins. IgE to the group 1 and 2 allergens appears to be very dominant in the allergic response of children. It has been shown that children attending an allergy clinic had strong IgE

binding to Der p 1 and 2 and sporadic responses to 1-3 additional allergens, whereas adults presenting at a clinic for allergy diagnosis had lower IgE binding to Der p 1 and 2 but recognised a diverse array of allergens (O'Brien and Thomas 1994). Similar phenomenon with bands equivalent to Der f 2 predominating in very young patients have also been demonstrated (Shibasaki and others 1994). While IgE binding to the group 1 and 2 allergens is high, the responses to other allergens must be considered. The report by van der Veen et al (van der Veen and others 2001) which showed that whole extract induced far stronger late phase reactions than a mixture of Der p 1 and 2, despite a similar early response, is evidence for this. Knowledge on major or important mite allergens has to be accumulated to elucidate their crucial roles in allergen sensitisation and provocation, as well as to enable effective allergy diagnosis and vaccines to be developed.

Major allergens of *D. farinae* to dogs are of 60-70 kDa and 90-109 kDa on SDS-PAGE (Noli and others 1996; McCall and others 2001; Nuttall and others 2001b), and they have recently been identified and characterised as Der f 18 (Weber and others 2003) and Der f 15 (McCall and others 2001) respectively. The majority of dogs with IgE specific for *D. farinae* also have IgE to Der f 15 chitinase. In one study, 95% (40 of 41) of naturally sensitised atopic dogs and 100% of experimentally sensitised laboratory beagles had positive IgE response for the high molecular weight 98/109 kDa allergen (McCall and others 2001). Der f 18-specific IgE was detected in 57% to 77% of *D. farinae*-sensitised dogs (Weber and others 2003). Both Der f 15 and Der f 18 are homologous to chitinase molecules and are both localised to the mite gut, suggesting a role in digestion other than moulting (McCall and others 2001; Weber and others 2003).

Most previous studies demonstrated little to no IgE binding to either low molecular weight allergens in crude *Dermatophagoides* extracts or purified group 1 and 2 allergens in dogs (Shaw S.C. 2000; McCall and others 2001; Nuttall and others 2001b). However, a study from Japan reported that at least 38% of atopic dog sera recognised Der p 1 and Der p 2, and 44% of samples recognised Der f 1 and Der f 2 in ELISAs and dot blots using monoclonal anti-canine IgE (Masuda and others 1999).

It might be possible that the spectrum of reactive allergens may differ between Japanese and European or US dogs (Nuttall and others 2006). The discrepancy could also arise from impurity of the allergen preparations (Le Mao and others 1998) and specificity and sensitivity of different antisera (Steward and Male 1998).

Identification and characterisation of the full repertoire of mite allergens will be an important step toward understanding their decisive roles in allergic sensitisation, improving diagnostic agents, and developing immunotherapeutic vaccines. Why humans and dogs respond to different allergenic components of house dust mites remains to be determined.

1.7.2 IgG and IgG subclasses

Immunoglobulins (Ig) are effector molecules of the humoral immune response and production of an Ig specific for a foreign molecule is the primary event responsible for recognition of an antigen. They are classified into one of five heavy (H) chain isotypes: IgM, IgG, IgE, IgD or IgA and in most species IgG antibodies can be further subdivided into four subisotypes (or subclasses) (Table 1.4). Each subisotype has a distinct effector function, such as the ability to cross the placenta, activate complement, respond to different types of antigen or bind to Fc receptors (Table 1.5). The IgG subclasses in humans are named in order of their abundance in serum, with IgG1 being the most abundant.

Table 1.3 IgE binding frequency to house dust mite allergens in humans[1]

Group	Species[2]	IgE binding[3]
1	Dp, Df, Dm, Ds, Em	80-100
2	Dp, Df, Ds, Em, Ld, Tp, Gd, As	80-100
3	Dp, Df, Ds, Em	16-100
4	Dp, Em	40-46
5	Dp, Bt, Ld	50-70
6	Dp, Df	40
7	Dp, Df, Ld	50
8	Dp	45
9	Dp	90
10	Dp, Df	50-95
11	Df, Bt	80
12	Bt	50
13	Bt, Ld, As	10-23
14	Df, Dp, Em	90
15	Df	70
16	Df	35
17	Df	35
18	Df	60
19	Bt	10

[1] based on the table in Thomas and others 2002.

[2] Allergen described for the species designated by initials: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Euroglyphus maynei*, *Dermatophagoides siboney*, *Dermatophagoides microceaus*, *Lepidoglyphus destructor*, *Blomia tropicalis*, *Tyrophagus putrescentiae*, *Glycophagus domesticus*, *Acarus siro*.

[3] Binding frequency (% patients, variation due to patient selection).

Table 1.4 IgG subclasses in various species

Species	Human	Dog	Mouse	Rat	Rabbit	Chicken
Subclass	IgG1	IgG1	IgG1	IgG1	IgG	IgG
	IgG2	IgG2	IgG2a	IgG2a		
	IgG3	IgG3	IgG2b	IgG2b		
	IgG4	IgG4	IgG3	IgG2c		

Table 1.5 Properties of human IgG subclasses[1]

	IgG1	IgG2	IgG3	IgG4
Physiochemical properties				
Heavy chain	γ1	γ2	γ3	γ4
Molecular mass (kDa)	146	146	170	146
Amino acids in hinge region	15	12	62	12
Inter-heavy chain disulfide bonds (in hinge region)	2	4	11	2
Susceptibility to proteolytic enzymes	++	+/–	+++	+
Biological properties				
Adult serum concentration (g/l) (mean)	4.9-11.4 (6.98)	1.5-6.4 (3.8)	0.20-1.10 (0.51)	0.08-1.40 (0.56)
Half-life (days)	21	20	7	21
Placental transfer	+++	+	++	+/–
Classical pathway of complement activation	++	+	+++	–
Alternative pathway of complement activation	–	–	–	–
Reactivity to proteins	++	+/–	++	+/–
Reactivity to polysaccharides	+	++	(–)	(–)
Reactivity to allergens	+	(–)	(–)	++
Binding to FcγI (CD64)	++	–	+++	+
Binding to FcγII (CD32)	++	([2])	+++	–
Binding to FcγIII (CD16)	++	–	++	–
Functional valency	2	2	2	1

[1]: Meulenbroek and Zeijlemaker 1996

[2]: FcγRII allotype dependent

The IgG subclass distribution in specific antibody responses has been found to vary with structure of the antigen (nature of carrier, number and nature of the epitopes, physicochemical properties), its dose and route of entry, as well as with genetic constitution of the host (Meulenbroek and Zeijlemaker 1996). Whereas antibodies against bacterial and viral protein antigens such as tetanus toxoid or outer-membrane components, which are T cell-dependent antigens, can be detected in all four IgG subclasses, IgG1 is the prevailing isotype and IgG2 generally provides only a marginal contribution (Ferrante and others 1990). On the other hand, IgG antibodies against polysaccharide antigens, which are usually T cell-independent, generally exhibit a much more pronounced subclass distribution. It has been shown that immunisation with encapsulated bacteria (e.g.: *Haemophilus influenzae* and *Neisseria meningitidis*) leads to an almost exclusive IgG2 anti-polysaccharide response (Siber and others 1980). Repeated, long-term antigenic stimulation with T cell-dependent antigens may lead to a marked IgG4 antibody response (Aalberse and others 1983).

The binding of IgG subclasses to antigen can result in direct inactivation of infectious agents by blocking of functional sites with enzymatic or receptor binding activity. More importantly IgG antibodies aggregated by antigen can interact with other components of the immune system by either activating the complement cascade or by binding to Fcγ receptors on various cell types (Clark 1995; Male and others 1996; Janeway and others 1999). Both of these processes can assist in the opsonisation of antigen and in the triggering of inflammation and the enhancement of an immune response against an infectious agent (Male and others 1996; Janeway and others 1999). The four IgG subclasses differ from each other with respect to their effector function (Table 1.5). This difference is related to differences in structure, notably with respect to the interaction between the variable, antigen-binding Fab-fragments and the constant Fc fragment. In particular, the length and flexibility of the hinge region are different. The capacity of the four human IgG subclasses to activate complement via the classical pathway decreases in the order: IgG3 > IgG1 > IgG2 > IgG4 (Meulenbroek and Zeijlemaker 1996). Phagocytosis is initiated by an interaction between the Fc fragment of the Ig and receptors, and Fc receptors for IgG

(FcγR) are expressed primarily on effector cells of the immune system including macrophages, monocytes, myeloid cells and dendritic cells. The four human IgG subclasses show differences in their interaction with FcγR's. Binding of the Fc part of IgG to a FcγR is instrumental in the induction of the cell's effector function; therefore, FcγR's play a key role in bridging IgG antibody activity and cellular effector mechanisms. The latter comprise phagocytosis, endocytosis, antibody-mediated cellular cytotoxicity, release of a range of inflammatory mediators, antigen presentation and clearance of immune complexes. Moreover, since several FcγR-bearing cell types have the capacity to present antigens to T lymphocytes, FcγR-induced phagocytosis also plays a role in antigen presentation and amplification of the immune response. The ability of the four IgG subclasses to initiate opsonisation via binding to FcγRI and FcγRII decreases in the order: IgG3 > IgG1 >> IgG4 and IgG3 > IgG1 > IgG2 respectively (Meulenbroek and Zeijlemaker 1996). The binding of IgG subclasses to FcγRIII is restricted to IgG1 and IgG3 (Huizinga and others 1989; Parren and others 1992).

The role of IgG antibody in atopic dermatitis is controversial and paradoxical. Evidence supporting and refuting the involvement IgG and IgG subclasses in AD will be discussed in detail in chapters 3 and 4.

1.8 The role of T-cells and cytokines in atopic dermatitis

1.8.1 Recruitment and activation of naïve T cells

T cell responses are initiated in the T cell areas of secondary lymphoid organs where naïve T cells encounter antigen-loaded dendritic cells (Banchereau and Steinman 1998). Skin-selective homing for effector and memory T cells represents an immunologic event in the pathogenesis of AD. The cutaneous lymphocyte-associated antigen (CLA) is a cell surface molecule preferentially found on T lymphocytes present in the skin. CLA interacts with the vascular receptor E-selectin, an adhesion molecule that is induced in endothelial cells under inflammatory conditions (Picker and others 1991), which forms the basis of the selective and

immediate recruitment of CLA+ T cells into the skin on invasion of allergens (Trautmann and others 2001; Akdis and others 2002).

The expansion and differentiation of naïve (CD45RA+ in humans) $CD4^+$ T cells is triggered by the binding of allergen/MHCII complexes on antigen presenting cells (APCs) (i.e. dendritic cells) to allergen-specific TCR/CD3 complexes in combination with costimulators. The binding between the T cell surface molecule CD28 with costimulatory molecules B7-1 (CD80) and B7-2 (CD86) expressed on activated APCs as well as the interaction of CD40L on T cells with CD40 on APCs provides essential signals for differentiation into activated (CD45RO+ in humans) $CD4^+$ T cells (Delves and Roitt 2000). When an immune response is initiated, naïve helper T cells produce IL-2 and proliferate. After entering the cell cycle, progeny become competent to produce effector cytokines such as IFN-γ (the signature of Th1 cells) or IL-4, IL-5 and IL-13 (the signature of Th2 cells) (Bird and others 1998). The propensity of an individual to mount a Th1 or Th2 response depends upon several factors, including the cytokine environment in which T-cell development takes place, the genetic background of the host, pharmacologic factors, and the costimulatory signals involved in T-cell activation.

1.8.2 T lymphocyte subsets

Two distinct $CD4^+$ T cells (helper T cells) subsets were first described in a detailed analysis of murine T-cell clones; one clone produced IL-2, IFN-γ, tumor-necrosis factor (TFN)-α, lymphotoxin-α and the other produced IL-4, IL-5, IL-6 and IL-13 (Mosmann and others 1986; Abbas and others 1996). These two populations were termed Th1 and Th2 cells, respectively. Since then Th1 and Th2 cells have also been found in humans and other species (Romagnani 1991; Maggi 1998).

Although individual cells may exhibit complex and quite heterogeneous patterns of cytokine production, it is customary to define $CD4^+$ T cells that have differentiated to produce IL-4 but not IFN-γ as Th2 (or Th2-like) cells and those that produce IFN-γ but not IL-4 as Th1 (or Th1-like) cells (Maggi 1998). Each T-cell subset produces

cytokines that serve as its own autocrine growth factor and promote differentiation of naïve T cells to that subset. Also, the two subsets produce cytokines that cross-regulate each other's development and activity (Fiorentino and others 1989; Fitch and others 1993). The net result of cytokine-mediate self-amplification and cross-regulation is that once a T-cell immune response begins to develop along one pathway, namely Th1 or Th2, it tends to become progressively polarised in that direction (Abbas and others 1996).

Recently, another T-cell subset that can inhibit T cell responses *in vitro* and *in vivo* and can prevent and ameliorate autoimmunity in several animal models has been described (Sakaguchi and others 1995; Shevach 2002). These cells have been named as T regulatory (Treg) cells. Natural Treg cells and adaptive Treg cells have been described, and both are antigen-specific, but they exert their regulatory activity in a non-specific way, which may be based on cell-to-cell contact, or the release of immuno-regulatory cytokines, such as transforming growth factor (TGF)-β or IL-10 (Chen and others 1994; Roncarolo and others 2001; Bluestone and Abbas 2003).

1.8.2.1 Th1 responses

Th1 cells secret IFN-γ, IL-2 and tumor necrosis factor (TNF)-α, which promote macrophage activation, production of opsonising and complement-fixing antibodies, antibody-dependent cell cytotoxicity (ADCC) and delayed-type hypersensitivity (DTH) reactions (Mosmann and Coffman 1989; Abbas and others 1996). The principal function of Th1 cells is to elicit phagocyte-mediated defence against infections, because Th1 cytokines promote the ability of macrophages to phagocytose as well as destroy microbes (Abbas and others 1996). However, if the Th1 response is not effective in eradicating infectious agents or excessively prolonged, it may become dangerous for the host, due to both the activity of cytotoxic cytokines and the strong activation of phagocytic cells (Maggi 1998). Some Th1 cells acquire cytolytic capacity, and the cytokines produced by Th1 cells, notably IL-2 and IFN-γ, promote the differentiation of $CD8^+$ T lymphocytes into

active cytotoxic cells. This is another way in which the Th1 subset participates in the elimination of intracellular microbes (Abbas and others 1996).

The signature cytokine of Th1 cells, IFN-γ, has two key functions. First, it enhances the microbicidal function of macrophages by stimulating the synthesis of phagocyte oxidase and inducible nitric oxide synthase (iNOS) to kill phagocytosed microbes (Hibbs, Jr. and others 1988; Jungi and others 1996). Second, IFN-γ stimulates B cells to produce certain IgG subclasses, such as IgG2a in mice (Finkelman and others 1988; Coffman and others 1993), which bind to high-affinity Fcγ receptors and complement proteins and are therefore the principal antibodies involved in the opsonisation and phagocytosis of particulate microbes. IFN-γ can also up-regulate expression of class I and class II MHC molecules and costimulators on APCs (Restifo and others 1993; Seliger and others 2000). Finally, it can also inhibit switching to IL-4-dependent isotypes, such as IgE and IgG1 in mice, or its homologue, IgG4, in humans (Coffman and others 1993). The net effect of these activities of IFN-γ is to promote macrophage-rich inflammatory reactions while inhibiting IgE-dependent eosinophil-rich reactions.

IL-12 stimulates the differentiation of $CD4^+$ helper T lymphocytes into IFN-γ-producing Th1 cells and the equilibrium between IL-12 and IL-4 is important for the balance between Th1 and Th2 responses. Activated mononuclear phagocytes and dendritic cells are the physiologically most relevant producers of IL-12 (Chehimi and Trinchieri 1994). IL-12 induces IFN-γ production from resting and activated NK and T cells (Kobayashi and others 1989; Chan and others 1991; Chehimi and Trinchieri 1994). It has an important role in the host resistance to infection, in particular to intracellular pathogens, by activating macrophages through induction of IFN-γ from NK and T cells and by enhancing cell-mediated immune responses, dependent on Th1 cell development. It also can enhance the cytolytic functions of activated NK cells and $CD8^+$ cytolytic T lymphocytes (CTLs). Being produced during early innate immune reactions against intracellular microbes and stimulating adaptive immune responses that protect the host against these microbes, IL-12 is an important link between innate and adaptive immunity (Abbas and Lichtman 2003).

IL-2, produced by T cells on antigen recognition, is responsible for the proliferation and differentiation of antigen-specific cells (Blackman and others 1986; Farrar and others 1986). It exerts its actions through the binding of high-affinity receptors (IL-2R) in an autocrine, paracrine and perhaps endocrine fashion (Smith 1988). Non-polarised T cells can be expanded with IL-2 and retain the capacity to differentiate into either Th1 or Th2 when re-stimulated under polarising conditions (Sad and Mosmann 1994). However, repeated activation of $CD4^+$ T cells in the presence of IL-2 makes these cells sensitive to apoptosis by the Fas pathway (van Parijs and others 1996).

The major cellular source of TNF-α is activated mononuclear phagocytes, although antigen-stimulated T cells, NK cells and mast cells can also secrete this protein (Bradding and others 1994; Kobayashi and others 2000). The principal physiologic function of TNF is to stimulate the recruitment of neutrophils and monocytes to sites of infection and to activate these cells to eradicate microbes (Abbas and Lichtman 2003). In severe infections, TNF is produced in large amounts and causes systemic clinical and pathologic abnormalities (Abbas and Lichtman 2003). TNF is strongly pro-inflammatory and a disease-related increase in the number of cells expressing immunoreactivity for TNF in asthmatics was observed (Kobayashi and others 2000). TNF can also increase the activity of dendritic cells (Skok and others 1999) but inhibits mast cells and FcεRI expression (Rossi and others 1998).

1.8.2.2 Th2 responses

The principal effector function of Th2 cells is in IgE-mediated and eosinophil / mast cell-mediated immune reactions. IL-4, IL-5, IL-6 and IL-13, produced by Th2 cells, promote mast cell and eosinophil growth, differentiation and activation, and provide optimal help for IgE and non-opsonising and complement fixing IgG (Abbas and others 1996; Maggi 1998). In addition, some Th2-derived cytokines, such as IL-4 and IL-13 inhibit several classical macrophage functions (Mosmann and Coffman 1989) while IL-13 also induces adhesion molecules on endothelial cells (Thornhill

and others 1990; Howells and others 1991; Sironi and others 1994; Till and others 1997). Th2 responses are less damaging to the host under normal circumstances; furthermore, Th2 cells may appear late in immune responses, and serve to limit the injurious consequences of Th1-mediated protective immunity (Abbas and others 1996).

IL-4 is the major inducer of B-cell switching to IgE production and is therefore a key initiator of IgE-dependent, mast cell-mediated reactions (Galli 1993). It is essential for the development of Th2 cells from naïve $CD4^+$ T cells and functions as an autocrine growth factor for differentiated Th2 cells (Seder and Paul 1994; Paul and Seder 1994). IL-4 exerts anti-inflammatory action by antagonising the macrophage-activating effects of IFN-γ and thus inhibits cell-mediated immune reactions (Xiao and others 2003).

IL-5 is the principal eosinophil-activating cytokine (Wardlaw and others 1995) and serves as the link between T cell activation and eosinophilic inflammation. The major actions of IL-5 are to activate mature eosinophils and stimulate the growth and differentiation of eosinophils (Lopez and others 1988; Sanderson 1992; Rothenberg and Hogan 2006), which are crucial in late phase inflammatory responses. IL-5 also supports IgE synthesis by IL-4 stimulated B cells and enhances basophil histamine release (Lalani and others 1999).

In relation to eradication of helminth infection, the two main Th2 cytokines, IL-4 and IL-5, function in concert: IL-4 stimulates production of IgE, which opsonises helminth and binds eosinophils, and IL-5 activates the eosinophils to destroy the parasites (Abbas and Lichtman 2003).

IL-13 is another important Th2 cytokine and shares many of its biologic activities with IL-4 because IL-4R and IL-13R complexes share the IL-4Rα chain required for signal transduction. However, it is unable to drive the differentiation of naïve $CD4^+$ T cells into a Th2 phenotype, and it does not support the proliferation of activated T cells either (Sornasse and others 1996). It has been shown in mouse models that IL-

13 is a central mediator in allergic asthma (Wills-Karp and others 1998; Grunig and others 1998) and a key cytokine for Th2 cell-mediated reactions in the lung (Chiaramonte and others 1999). In humans, IL-13 may affect T-cell functions and Th1-cell differentiation indirectly through its down-regulatory effects on the production of pro-inflammatory cytokines, particularly of IL-12 by monocytes, which direct Th1 development (de Vries 1998). Human IL-13 also plays an important role in the regulation of Ig class switching, and enhances IgE production (Chomarat and Banchereau 1997). Recombinant canine IL-13 can stimulate 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.

1.8.2.3 Regulatory T cells

Two groups of Treg cells, natural Treg cells and adaptive Treg cells, have been described (Bluestone and Abbas 2003). Natural Treg cells emerge from the thymus during ontogeny as a population already dedicated to suppressing the response against self-antigens (Sakaguchi and others 1995; Shevach 2002) and are characterised by their $CD4^{+}$ $CD25^{+}$ phenotype. These cells have been suggested to develop under the control of the transcription factor FoxP3 (Hori and others 2003). Adaptive Treg cells also derive from the thymus but acquire suppressive activity in the periphery and are devoted to regulate the response not only against self but also against non-self antigens (Bluestone and Abbas 2003). Two distinct subsets of adaptive Treg cells have been reported: type 3 T helper (Th3) cells that can be induced by the oral administration of antigen (Chen and others 1994) and T regulatory 1 (Tr1) cells, which are induced by the administration of antigen in the presence of IL-10 (Roncarolo and others 2001). Treg cells appear to exert their regulatory effects via expression of inhibitory cell-surface molecules and/or by the production of immuno-regulatory cytokines, such as IL-10 and TGF-β (Chen and others 1994; Roncarolo and others 2001).

IL-10 was originally termed cytokine synthesis inhibitory factor because it was identified as a soluble factor able to inhibit cytokine secretion from mouse Th1 cells (Moore and others 1990). IL-10 has anti-inflammatory and suppressive effects on most haematopoietic cells and participates in induction of peripheral tolerance (Moore and others 2001). It indirectly suppresses T-cell responses by potently inhibiting the antigen-presenting capacity of APCs, including dendritic cells, Langerhans cells and macrophages. IL-10 down regulates expression of MHC II and co-stimulatory molecules such as CD54, CD80 and CD86 (Moore and others 2001). IL-10 also potently inhibits the secretion of cytokines and chemokines by macrophages, neutrophils, eosinophils, mast cells, keratinocytes, endothelial cells, as well as APCs, which influence T-cell differentiation, proliferation and migration (Moore and others 2001). In addition, IL-10 directly regulates T cells by inhibiting their ability to produce IL-2, TNF-α (de Waal Malefijt and others 1993) and IL-5 (Schandene and others 1994) and to proliferate (Bejarano and others 1992; Taga and others 1993). On the other hand, IL-10 also has many immuno-stimulatory effects and can enhance the survival of human B cells and their differentiation into plasma cells (Moore and others 2001). Furthermore, IL-10 inhibits T-cell apoptosis (Cohen and others 1997) and has clear stimulatory effects on $CD8^+$ T cells as it increases their proliferation and cytotoxic activity (Groux and others 1998).

T cells are influenced by TGF-β at all stages of their development (Letterio and Roberts 1998), and TGF-β inhibits T-cell proliferation, cytokine production and cytotoxicity. However, it also has positive effects on T-cell function via inhibition of T-cell apoptosis (Zhang and others 1995), enhancement of naïve T-cell proliferation and induction of cytokines (Cerwenka and others 1994; Cerwenka and others 1996). The effects of TGF-β on APCs can be stimulatory or inhibitory depending on the presence of other cytokines, their state of maturation and levels of receptor expression (Letterio and Roberts 1998). TGF-β is required for the differentiation of Langerhans cells and can increase their viability (Borkowski and others 1996). Low concentrations of TGF-β induce chemotaxis, expression of a variety of cytokines and phagocytic activity of monocytes (Letterio and Roberts 1998). Additionally, TGF-β suppresses IFN-γ production, decreases MHC II expression and limits the production

of nitric oxide and reactive oxygen intermediates by activated macrophages (Bogdan and Nathan 1993; Letterio and Roberts 1998).

1.8.2.4 T cell subsets cytokine profiles

Whilst mouse $CD4^+$ T-cell clones can be classified into distinct populations on the basis of their patterns of cytokine production (Romagnani 1995), in human cells this is much less so. Individual cells may exhibit complex and quite heterogeneous patterns of cytokine production, with various combinations of IL-2, IL-4, IL-5, IFN-γ, IL-10 and TGF-β (Abbas and others 1996; Grewe and others 1998), suggesting that the transcription and expression of different cytokine genes are regulated independently. However, Th1 (IFN-γ-secreting) and Th2 (IL-4-secreting) polarised responses can still be distinguished and the degree of polarisation and heterogeneity of T lymphocytes may reflect the nature of the antigenic and environmental stimuli to which the cells have been exposed (Abbas and others 1996; Maggi 1998) although the expression of IL-2, IL-6, IL-10 and IL-13 is less restricted in humans (Romagnani 1991; Romagnani 1995). In the absence of clear polarising signals, $CD4^+$ T cell subsets with a less restricted lymphokine profile than Th1 or Th2 cells, designated Th0, usually arise, that mediate intermediate effects depending on the ratio of lymphokines produced and the nature of the responding cells (Maggi and others 1988; Paliard and others 1988; Mosmann and Coffman 1989; Street and others 1990). Mixed (Th0) cytokine patterns are most noticeable early after lymphocyte activation (Kelso 1995). The cytokine response of the single Th0 cells can remain unrestricted or further differentiate into a polarised Th1 or Th2 pathway under the influence of micro-environmental signals or because of a particular genetic background (Seder and Paul 1994).

1.8.2.5 Antagonism of Th1 and Th2 responses

While IL-4 inhibits the development of Th1 cells, IFN-γ inhibits the development of Th2 cells (Romagnani 1994; Abbas and others 1996). Moreover, the expression of transcription factor GATA-3, which allows the differentiation of Th2 cells, inhibits

Th1 development; conversely, expression of transcription factor T-bet, which allows the differentiation of Th1 cells, inhibits Th2 development (Rengarajan and others 2000). Transcription factors expressed by, and cytokines released from, Th1 or Th2 cells exert a mutual antagonistic effect on the development of the other subset.

1.8.3 The Th1/Th2 hypothesis in atopic dermatitis

1.8.3.1 Th2 cytokines in human atopic dermatitis

A considerable number of studies have associated human AD with Th2 polarisation. Th2 cells produce IL-4 and IL-13 which stimulate IgE and IgG4 antibody production, and IL-5 which recruits and differentiates eosinophils, thus explaining why the mast cell/eosinophil/IgE-producing B cell triad is involved in the pathogenesis of allergy (Romagnani 1994). The great majority of allergen-specific T-cell clones generated from peripheral blood lymphocytes of atopic donors express a Th0/Th2 phenotype, with high production of IL-4 and IL-5 and no or low production of IFN-γ, whereas T cell clones specific for bacterial antigens derived from the peripheral blood from the same atopic donor show a prevalent Th1/Th0 phenotype (Wierenga and others 1990; Kapsenberg and others 1991; Yssel and others 1992; Ebner and others 1993). Skin-derived *Dermatophagoides pteronyssinus* (DP)-specific Th2-like $CD4^+$ T cell clones were generated after contact challenge with DP (van Reijsen and others 1992). Furthermore, the Th2 hypothesis in atopy is confirmed by studies on the effect of allergen-specific immunotherapy (IT) on the cytokine profile of T cells. Decreased production of IL-4 and increased production of IFN-γ was observed in bee venom-sensitised patients treated with specific IT (Jutel and others 1995; McHugh and others 1995), and successful immunotherapy is associated with a shift from the Th2 seen in allergy towards a Th1 response leading to a decline in allergen-specific IgE and an increase in allergen-specific IgG production (Renz 1995; Smith and Sly 1998). Most importantly, however, production of the immunosuppressive / immunoregulatory cytokine IL-10 is also induced leading to T cell tolerance and prevention of tissue inflammation (Bellinghausen and others 2001).

Allergen-specific Th2 clones from PBMCs and the skin of atopic patients have been isolated in various studies (Mudde and others 1992; Neumann and others 1996; Punnonen and others 1997; Bohle and others 1998), and these Th2 cell clones secrete large amounts of IL-4 and IL-5 but under-express IFN-γ. In contrast, stimulation of PBMCs with non-allergenic antigens expands non-polarised or Th1 clones (Romagnani 1998). There is a relatively consistent difference in the balance or ratio of Th1 to Th2 cytokines in atopics vs. non-atopics (Smith and Sly 1998).

Several studies proposed that Th2-type cells play a key pathogenetic role in AD (Plaut 1990; Kay and others 1991; Bos and others 1992). The frequency of Th2 clones isolated from the skin usually exceeds that from PBMCs (Grewe and others 1998), suggesting there is either selective recruitment of Th2 cells or local *in situ* Th2 differentiation in the skin. However, by analysing T-cell cytokine expression during the development of atopy patch test lesions, a biphasic expression pattern is observed (Grewe and others 1995; Thepen and others 1996). At relatively early time points (24 h after allergen application), increased expression of IL-4 mRNA and protein was observed, after which (48, 72 and 96 hours after allergen application) IL-4 expression declined to background levels. By contrast, increased IFN-γ mRNA expression was not detected in 24 h patch-test lesions, but was strongly over-expressed at later time points (48-72 h). These observations may be best explained by a model in which the initiation of AD is driven by activation of Th2-type cells, whereas the chronic inflammatory reaction is dominated by a Th1-type response (Grewe and others 1998).

It has also been found that early immunisation with the offending allergen is critical for the initiation of immunological changes resulting in the development of atopy. The selective development of a Th2 cytokine profile in high-risk children who develop atopy is due to increased production of Th2 cytokines, possibly caused by impaired allergen-induced IFN-γ production in the neonatal period (van der Velden and others 2001). Furthermore, the decreased allergen-induced IL-10 levels observed in the atopic children at 12 months of age may result in a lack of down-regulation of the inflammatory process (van der Velden and others 2001).

1.8.3.2 Th1 cytokines in human atopic dermatitis

Th1-type cytokines are also expressed in atopic patients. Chronic AD skin lesions have significantly fewer IL-4 and IL-13 mRNA-expressing cells, but greater numbers of IL-5, IL-12 and IFN-γ mRNA-expressing cells than in acute AD (Grewe and others 1998; Leung and others 2004). Moreover, increased *in situ* expression of the Th2-type cytokines was observed together with increased expression of the Th1-type cytokine IFN-γ within identical involved skin areas of the same patients (Grewe and others 1994; Ohmen and others 1995). Furthermore, peak levels of IFN-γ mRNA expression was present at 48-72 hours after allergen application in atopy patch tests (Thepen and others 1996). Taken together, the data suggest that expression of Th2-type and Th1-type cytokines in AD is not mutually exclusive, and that both Th-cell subsets contribute to the pathogenesis of the disease (Grewe and others 1994; Ohmen and others 1995; Hamid and others 1996; Morita and others 1997).

1.8.3.3 Treg cytokines in human atopic dermatitis

In addition to mutual antagonism of Th1 and Th2 responses, regulation of the immune system is also carried out by the activity of other T-cell types, which have been named Treg cells. Treg cells are a highly heterogeneous family, which includes type 3 Th (Th3) cells, T regulatory 1 (Tr1) cells, and $CD4^+$ $CD25^+$ T cells. Th3 cells mainly produce TGF-β and their regulatory function is attributable to TGF-β-dependent mechanisms (Weiner 2001), whereas Tr1 cells are mainly able to produce IL-10, with or without TGF-β (Roncarolo and others 2001). In contrast, $CD4^+$ $CD25^+$ T cells do not produce cytokines and act via a contact-dependent mechanism (Suri-Payer and others 1998). Another feature of $CD4^+$ $CD25^+$ T cells is the expression of the transcription factor Foxp3 (Fontenot and others 2003).

In both experimental animal models and humans, genetic alterations which heavily hamper the development and/or the function of Treg cells can result in the appearance of multiple and severe inflammatory disorders, including allergy (Kanangat and others 1996; Chatila and others 2000; Curotto de Lafaille and others

2001). Suppressive cytokines of Tr1 cells, IL-10 and TGF-β, and receptors for these cytokines are abundantly expressed in the skin of AD patients whereas FoxP3$^+$ CD25$^+$ T cells are not present (Verhagen and others 2006). It seems that IL-10 produced by Treg cells acts mainly by shifting the allergen-specific antibody production from the dangerous IgE to the protective IgG4 isotype rather than by inducing a Th2-cell tolerance (Nouri-Aria and others 2004). It has been shown that IL-10-secreting Tr1 cells can inhibit the allergen-specific proliferation of IL-4-secreting Th2 as well as of IFN-γ-secreting Th1 cells. A previous study has also demonstrated an over-expression of IL-10 in atopic skin in humans (Ohmen and others 1995)

It has been proposed that the suppression of allergic disease following successful ASIT is mediated by the production of IL-10 by Treg cells (Akdis and others 1998a). TGF-β either secreted by or associated with the cell membrane of Treg cells is also an attractive candidate to inhibit allergic responses (Singh and others 2001). For both IL-10 and TGF-β, it may be critical that these cytokines are expressed in a particular temporal and/or cellular context to achieve optimal immunotherapy and minimise adverse effects (Verhagen and others 2006).

So far, the experimental evidence in favour of an important role of Treg cells on dampening allergen-specific Th2 responses is still poor and contradictory, due to the heterogeneity of Treg cells and to the incomplete knowledge of their origin and function. In contrast, solid data published over the last few years has shown that allergen-specific Th2 responses are subjected to the negative control of Th1-polarising cytokines (IFN-γ, IL-12), which are produced in response to chronic and repeated stimulation of Toll-like receptors (TLRs) present on the cells of the innate immune system (Braun-Fahrlander and others 2002; Romagnani 2004a). Therefore, it is more likely that IL-12 or IFN-α-producing dendritic cells and IFN-γ-producing NK cells, and Th1 cells themselves, play even more important regulatory effects on allergen-specific Th2 responses than classic Treg cells (Romagnani 2004b; Romagnani 2006).

1.8.3.4 Cytokine expression in canine atopic dermatitis

Limited studies have been performed investigating the production of Th1 and Th2 type cytokines in canine atopic dermatitis. Expression of T cell subset cytokines in canine AD will be discussed in detail in chapter 6.

1.8.4 CD8$^+$ cells in atopic dermatitis

Distinct subpopulations of CD8$^+$ T cells (Tc1 / Tc2 / Tc0 cells) can be compartmentalised along broadly similar lines to CD4$^+$ T cells – with IFN-γ secretion representing Th1 / Tc1 cells, IL-4 secretion representing Th2 / Tc2 cells and an unrestricted cytokine profile representing Th0 / Tc0 cells (Croft and others 1994; Noble and others 1995; Sad and others 1995; Kemeny 1998). The role of CD8$^+$ T cells in atopic dermatitis is still unclear and probably complex. Some studies suggest a suppressive function of IFN-γ-producing CD8$^+$ T cells with inhibition of IgE production and consequent down-regulation of allergic sensitisation (McMenamin and Holt 1993; Renz and others 1994). In contrast, under certain circumstances Tc2 CD8$^+$ T cells are required for IgE responses and have been shown to promote IgE class switching *in vitro* (Erard and others 1993; Le Gros and Erard 1994). Th2-like CD8$^+$ T cells are present at sites of allergic inflammation (Gemou-Engesaeth and others 1997; Ying and others 1997) but it is not clear if sufficient antigen is available to activate them or the extent to which they contribute to allergic inflammation (Kemeny 1998).

1.9 Immunological changes during allergen-specific immunotherapy

Allergen-specific immunotherapy (ASIT) is the practice of administering gradually increasing quantities of an allergen extract to an allergic subject to ameliorate the symptoms associated with subsequent exposure to the causative allergen (Bousquet and others 1998). It is a widely used therapeutic option for allergic diseases and is an effective treatment of allergic rhinitis and venom anaphylaxis (Theodoropoulos and Lockey 2000). The goal of immunotherapy is to alter the inappropriate

immunological reactivity to innocuous environmental substances. Whereas the symptoms of immediate and delayed type allergic reactions can be ameliorated by various pharmacologic treatments, ASIT represents the only curative approach for specific allergies (Akdis and Blaser 2000).

1.9.1 History of allergen-specific immunotherapy

Desensitisation and hyposensitisation, the terms most commonly used previously for many years, refer to a reduction or loss of sensitivity (Anderson 2000), but these terms do not reflect the mechanisms believed to be important, nor the agents being used. Allergen immunotherapy as designated by a WHO position paper (Bousquet and others 1998) or allergen-specific immunotherapy proposed by the American College of Veterinary Dermatology Task Force on Canine Atopic Dermatitis (Griffin and Hillier 2001b) are more appropriate terms.

Almost a century ago, long before the immunopathological mechanisms that underlie IgE-mediated allergy were understood, Noon (1911) immunised patients suffering from pollen-induced hay fever with subcutaneous injections of pollen extracts (Noon 1911). Although this was based on the erroneous belief that seasonal hay fever might be caused by a grass-pollen toxin, successful outcomes were recorded and protection was found to last for at least one year after the treatment was discontinued (Noon 1911; Valenta 2002). The first successful treatment using ASIT in a dog with seasonal hay fever was also reported long ago (Wittich 1941). Since then, numerous studies have been performed to analyse the underlying immunological and molecular mechanisms as well as environmental factors and genetic background that influence the development of allergy. However, due to the exceedingly complex interaction of numerous immunological cells, molecules, cytokines and the influence of the microenvironment, the immunological mechanisms by which ASIT achieves clinical improvement are still incompletely understood.

The differentiation, phenotype and activity of allergen-specific T cells are controlled by various allergen-specific factors, including antigen concentration (Secrist and

others 1995), antigen conformation (Akdis and others 1998b) and the site of antigen exposure (Constant and others 2000). Together with the finding that the profile of allergens and epitopes that are recognised by IgE does not change substantially during the natural course of allergic disease (Ball and others 1999a), modulating allergen-specific T cells using specific antigen is an excellent basis for the antigen-targeted immunotherapy of allergy (Valenta 2002).

The most effective and frequently used form of allergen immunotherapy clinically is injection immunotherapy (Bousquet and others 1998). It involves repeated subcutaneous injections of increasing doses of allergen extract. Numerous clinical studies have documented the efficacy of immunotherapy, but the underlying immunological mechanisms remain a matter of controversy (Durham and Till 1998; Bousquet and others 1998).

1.9.2 Potential mechanisms of ASIT

Most research into immunotherapy mechanisms has examined the effect of subcutaneous immunotherapy rather than immunotherapy by alternative routes. Mechanisms might have a degree of heterogeneity, reflecting variability in the types of allergic diseases treated, different patient populations, the use of different adjuvants, and the route, dose, and duration of treatment (Till and others 2004). Proposed possible potential mechanisms of ASIT are summarised in Table 1.6.

1.9.2.1 Serum antibody responses

In 1935, more than 30 years before the characterisation of IgE antibodies, Cooke et al provided the first insight into the mechanisms of injection immunotherapy by showing that allergic patients who were treated by ASIT developed antibodies which blocked allergen-induced inflammation (Cooke and others 1935). A few years later, the factor was identified as IgG antibodies, and due to their ability to inhibit immediate skin reactions to allergen provocation, were designated blocking antibodies (Loveless 1940).

Although quantitative changes in IgG antibody do not always correlate with clinical protection (Djurup and Malling 1987; Muller and others 1989; Ewan and others 2006), recent findings demonstrated clear involvement of those antibodies. Immunotherapy induced IgG antibodies (mainly IgG1 and IgG4) compete with IgE for their binding sites on the allergen, reducing cross-linking of IgE on mast cells (Mothes and others 2003) and basophils (Garcia and others 1993), thus inhibiting release of histamine and other mediators. Blocking antibodies also strongly suppress allergen-specific T cell responses *in vitro* by reducing CD23-dependent IgE-facilitated allergen presentation by B cells to T cell clones, which might reduce Th2 activation and the subsequent release of Th2 cytokines (van Neerven and others 1999; Wachholz and others 2003).

It has been found that a 10-50 times lower threshold amount of antigen is required for the induction of IL-4 than for IFN-γ (Carballido and others 1994). Increasing antigen concentrations favours IFN-γ production by T cells, whereas IL-4 decreases at high antigen doses (Carballido and others 1994). Low antigen concentration and suboptimal antigenic peptide-binding capacity of MHC II molecules generate weak T-cell activation, resulting in IL-4 dominated Th2 cytokine pattern and IgE production (Akdis and Blaser 2000). Such regulatory effects of allergen concentration on cytokine secretion may also reflect a physiologic mechanism in ASIT in which repeated high allergen doses are injected over a longer period of time (Carballido and others 1993; Blaser and others 1998).

1.9.2.2 T-lymphocyte responses

1.9.2.2.1 Peripheral responses

Studies of patients being treated with venom or inhalant allergen immunotherapy which examined cells isolated from peripheral blood demonstrated a reduction in proliferative responses to allergen, with an overall deviation from a Th2 to Th1 response (Jutel and others 1995; Ebner and others 1997); but other studies have not

reproduced these findings (Wachholz and others 2002; Francis and others 2003). A possible explanation is that inhibition of peripheral T-cell proliferation and Th2 cytokine production is not the fundamental event in immunotherapy (Till and others 2004). In contrast, production of IL-10 in peripheral blood in response to immunotherapy is highly consistent (Bellinghausen and others 1997; Akdis and others 1998a). It has been demonstrated that allergen-induced IL-10 and TGF-β secretion by allergen-specific T cells occurs in parallel to a global suppression of Th2 proliferative responses and cytokine production (Jutel and others 2003).

Selective recruitment of allergen-specific T cells to allergic tissue sites in allergic patients has been identified via CXCR1 on $CD4^+$ cells, and CXCR1 expression on circulating $CD4^+$ T cells from patients receiving grass pollen immunotherapy is low or absent compared with symptomatic patients with allergic rhinitis (Francis and others 2004). The effect of immunotherapy on other T-cell chemokine receptors remains to be determined.

1.9.2.2.2 T-lymphocyte responses in tissue

It seems reasonable when dealing with allergy to various allergens that studies of the immunological changes within the allergic tissue are of greatest relevance (Till and others 2004). In grass pollen immunotherapy, clinical improvement is accompanied by a decrease in the size of late-phase skin responses (Varney and others 1993). Using *in situ* hybridisation detecting specific cytokine mRNAs in skin, increased IFN-γ and IL-2 mRNA-expressing cells are present although reduction in numbers of IL-4 or IL-5 mRNA expressing cells is not observed (Varney and others 1993). IL-12 mRNA expression is also increased after immunotherapy and correlates positively with IFN-γ mRNA expression and inversely with IL-4 expression (Hamid and others 1997). Increased allergen-dependent IFN-γ mRNA expression without reductions in IL-4 and IL-5 mRNA after immunotherapy are also documented in nasal mucosal biopsy specimens (Durham and others 1996). Changes in protein concentration are also consistent with cytokine mRNA level (Klimek and others 1999). IL-10 mRNA-expressing cells in the nasal mucosa of grass sensitive patients after immunotherapy,

and expression of IL-10 is allergen driven, as well as immunotherapy dependent (Nouri-Aria and others 2004).

1.9.2.2.3 IL-10 and regulatory T cells

Immunotherapy-induced IL-10 increase is recorded in various studies (Bellinghausen and others 1997; Akdis and Blaser 2001a; Francis and others 2003). The anti-allergic properties of IL-10 include modulation of IL-4-induced B-cell IgE production in favour of IgG4 (Jeannin and others 1998), inhibition of IgE-dependent mast cell activation (Royer and others 2001) and inhibition of eosinophil cytokine production and survival (Takanaski and others 1994). In human T cells IL-10 suppresses production of pro-allergic cytokines, such as IL-5 (Francis and others 2003), and is able to induce a state of antigen-specific hyporesponsiveness or anergy (Groux and others 1997). Intracellular IL-10 is significantly increased after 1 week of ASIT in the antigen-specific T cell population and activated $CD4^+$ cells (Akdis and others 1998a). After 4 weeks of therapy, intracytoplasmic IL-10 is also increased in monocytes and B cells, suggesting an autocrine action of T-cell-secreted IL-10 as a pivotal step in the induction phase of T-cell anergy (Akdis and others 1998a). However, due to lack of a unique marker that defines all cells with regulatory activity, the properties of T cells producing IL-10 after immunotherapy are contentious. There is no conclusive evidence to identify whether natural Treg cells or adaptive Treg cells (Th3 and/or Tr1) are actively involved in the up-regulation of IL-10 after ASIT. But the production of IL-10 and TGF-β by T cells represents an important component of successful immunotherapy and is a marker of successful down-regulation of allergen-specific T-cell responses after immunotherapy (Till and others 2004).

The biology of regulatory T-cell induction is relatively poorly understood, but recent evidence suggests that dendritic cells seem likely to play a critical role (Penna and others 2005). However, the involvement of tolerogenic dendritic cells in immunotherapy remains to be investigated.

1.9.2.3 B-lymphocyte responses

In untreated patients with birch pollen allergy, seasonal exposure to birch showed an increase in cells with surface expression of CD23, CD40 and HLA-DR at the same time IgE antibodies increased, whereas no such increases occurred in patients given immunotherapy (Hakansson and others 1998). Allergen-specific IgG antibodies induced by immunotherapy can disrupt formation of allergen-IgE complexes that bind to APC and facilitate allergen presentation (Wachholz and others 2003; Nouri-Aria and others 2004).

1.9.2.4 Inflammatory cytokines

The eosinophil and basophil stimulator IL-5 is down-regulated after ASIT (van Bever and others 1998; Oda and others 1998; Jutel and others 2003). *In vitro* degranulation of neutrophils after stimulation is higher in asthmatic patients than in healthy control subjects and returns to normal levels after immunotherapy (Monteseirin and others 2001). In nasal mucosal biopsy specimens from patients receiving grass pollen immunotherapy, the number of eosinophils is lower in treated patients, and both eosinophils and IL-5 correlate with symptoms after immunotherapy (Wilson and others 2001). Studies of serum cytokine levels for dust mite allergy demonstrate a reduction in IL-1β and TNF-α and augmentation of IL-2 and IL-6 after ASIT, which indicates a reduction in inflammatory responses (de Amici and others 2001).

1.9.2.5 Mast cells and basophils

It is possible that several different mechanisms are responsible for the observed long-term effects of injection immunotherapy and for the clinical observation that this form of allergen-specific immunotherapy can prevent the progression of allergic disease from less severe to more severe symptoms.

Table 1.6 Potential mechanisms of allergen-specific immunotherapy[1]

Targeted process	Mechanism	References
Immediate-phase reactions		
Immediate skin reactions (mast-cell degranulation)	Blocking IgG competing with IgE	(Loveless 1940)
Basophil degranulation	Blocking IgG competing with IgE	(Ball and others 1999a; Ball and others 1999b)
Mast-cell number	Reduction by unknown mechanism	(Durham and others 1999)
Mediator release from mast cells and basophils	Reduction by T-cell-derived IL-10 and IFN-γ	(Pierkes and others 1999)
Late-phase reactions		
IgE-mediated allergen presentation to T cells, T-cell activation and cytokine release	Blocking IgG competing with IgE	(van Neerven and others 1999)
T-cell proliferation	Generation of Treg cells; T-cell tolerance/anergy	(Rocklin and others 1980; Pierkes and others 1999) (Hoyne and others 1993; Briner and others 1993; Akdis and others 1996; Ebner and others 1997; Muller and others 1998; Hakansson and others 1998; Oldfield and others 2001; Akdis and others 2001b)
Th2 cytokine production	Reduction of T-cell-derived IL-4 and IL-13	(Briner and others 1993; Jutel and others 1995; McHugh and others 1995; Secrist and others 1995; Ebner and others 1997; Gabrielsson and others 2001)
Th1 cytokine production	Induction of Th1 cells	(Jutel and others 1995; McHugh and others 1995; Ebner and others 1997)
Eosinophil number	IL-5 reduction	(Wilson and others 2001)

[1]Adapted from Valenta (2002)

1.10 Aims and objectives of the studies in this thesis

Our current understanding of the immunopathogenesis of canine atopic dermatitis is relatively poor compared to humans and murine models. Until recently, most publications have concentrated on the clinical signs, the results of skin testing and IgE serology, and treatment options.

The aim of these studies was to investigate further some aspects of the immunological mechanisms that may be involved in canine atopic dermatitis. Two main areas were targeted for investigation:

1. The role of *D. farinae*-specific IgG and IgG subclasses in the disease.

This area has received relatively little study in the canine disease as most research has focussed on IgE. However, the role of IgG requires further clarification as the balance between IgE and IgG production may throw some light on the nature of the immunological responses generated to environmental allergens. Also, some investigators have claimed a specific role for subclasses of IgG in the pathogenesis of the disease (Willemse and others 1985b). IgG may also play a role in the mechanism of action of allergen-specific immunotherapy. To perform these studies, the aim was to develop and validate a semi-quantitative Western blot analysis system that would allow characterisation of the IgG response to separated proteins of *Dermatophagoides farinae*.

2. Further characterisation of cytokine profiles in atopic dogs.

Previous studies investigating the cytokine profiles in the skin of atopic dogs have yielded inconsistent results. The aim in these studies was to develop real time, quantitative RT-PCR techniques to measure mRNA transcripts for four cytokines representing different immunological classes: IFN-γ as an archetypal Th1 cytokine; IL-4 as an archetypal Th2 cytokine; TGF-β as an example of a regulatory (immunosuppressive) cytokine; and inducible nitric oxide synthase (iNOS) as a measure of the innate immune response. To develop these assays, the aim was to clone the gene for each of these cytokines, express it in a vector system and use the product to develop a standard curve in real-time PCR assays.

Chapter 2
Materials and Methods

2.1 Atopic and healthy dogs

2.1.1 Study population

Dogs diagnosed with atopic dermatitis were recruited from the dermatology clinic at the Royal (Dick) School of Veterinary Studies Hospital for Small Animals, Easter Bush Veterinary Centre, Roslin, Midlothian, EH25 9RG, UK (n=43) and from the Animal Dermatology Clinic, 2965 Edinger Avenue, Tustin, CA 92780, USA (n=15). Samples from UK dogs were either collected under Home Office licence or were residual to diagnostic use. Control samples were taken from healthy dogs presented for euthanasia by a local rescue centre to the Royal (Dick) School of Veterinary Studies Hospital for Small Animals (n=20). These dogs had behavioural problems and were assessed as being unsuitable for re-homing. Euthanasia was performed with intravenous administration of sodium pentobarbitone (Euthatal®, Merial Animal Health Ltd., Harlow, Essex, UK) at a dosage of 150 mg per kg body weight. All procedures on euthanised animals were carried after recognition of death, as assessed by cessation of heartbeat and respiration, absence of reflexes, including corneal and palpebral reflexes, and glazing of eyes.

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 skin lesions at the time of sampling. No topical or systemic anti-inflammatory medications had been given for at least three weeks (eight weeks for injectable depot preparations) prior to examination.

2.1.3 Inclusion criteria for the dogs with atopic dermatitis

The diagnosis of atopic dermatitis was based on a combination of consistent history of chronic seasonal or perennial pruritus that was responsive to glucocorticoids, typical clinical signs, exclusion of other causes of pruritic skin disease, and one or more positive reactions in an intradermal test or IgE serological assay (Willemse 1986). All the atopic dogs included in this study were tested positive to *D. farinae* either on intradermal testing or IgE serology. Ectoparasitic infestation was ruled out based on negative results of multiple skin scrapings, coat brushing or serological testing (IgG enzyme-linked immunosorbent assay (ELISA) for *Sarcoptes scabiei*) where necessary. All dogs were placed on a rigorous flea control programme for a minimum of six weeks using topical fipronil (Frontline®; Merial, Harlow, UK) or selamectin (Stronghold®; Pfizer Animal Health, Sandwich, UK). Dogs that experienced complete resolution of clinical signs after treatment were excluded.

All dogs underwent a six-week, home cooked or commercial diet trial. 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 adverse food reaction in dogs that clinically improved during 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 antimicrobial medication was given for at least three weeks (eight weeks for injectable depot preparations) before examination.

2.1.4 Allergen-specific IgE tests

2.1.4.1 Intradermal skin tests

Dogs with a presumptive diagnosis of atopic dermatitis were intradermally tested with 57 allergens (Table 2.1) as follows. Dogs were sedated with 0.15 mg/kg xylazine (Virbaxyl®; Virbac) intramuscularly. A rectangular area from the lateral flank was clipped and 0.05 ml of each allergen extract, 0.1 mg/ml histamine (positive control) and buffered saline (negative control) with 0.4% phenol diluent as preservative were injected intradermally via a 25-gauge needle. The degree of swelling, erythema and induration at each injection site were subjectively assessed after 20 min. 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 relevant (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, S. Colombo, K.L. Thoday or A. Neuber; Dermatology Clinic, University of Edinburgh Hospital for Small Animals or C.E. Griffin, R. Muse, W.S. Rosenkrantz, or C. Mendelsohn; Animal Dermatology Clinic, 2965 Edinger Avenue, Tusti, CA 92780).

2.1.4.2 Allergen-specific IgE serology

Serum samples from dogs that did not undergo intradermal skin tests, or in which skin tests were negative, were analysed using allergen-specific IgE serology (ASIgES) (FcεRIα-based ELISA, Allercept™, Heska Corporation, Fribourg, Switzerland). Allergens included in the test are listed in Table 2.2.

Table 2.1 Intradermal test allergens

Greer – Greer laboratories, Lenoir, NC, USA; Artu – Artuvetrin® Test; Artu Biologicals, Lelystad, The Netherlands; PNU – protein nitrogen units; NE – nitrogen equivalent.

Allergen extract	Source	Concentration
Controls		
Histamine	Artu	0.1 mg/ml
Diluent	Greer	n/a
Dust and dust mites		
Dermatophagoides pteronyssinus	Greer	1/1000 w/v
Acarus siro	Artu	100 NE/ml
Dermatophagoides farinae	Greer	1/1000 w/v
Tyrophagus putresceantiae	Artu	100 NE/ml
Euroglyphus maynei	Artu	100 NE/ml
House dust	Greer	1000 PNU/ml
Epithelials		
Human epithelia	Artu	10 µg/ml
Cat epithelia	Greer	100 µg/ml
Horse epithelia	Greer	500 PNU/ml
Sheep epithelia	Greer	500 PNU/ml
Mixed feathers	Artu	100 µg/ml
Cotton linters	Greer	1000 w/v
Insects		
American cockroach	Greer	500 PNU/ml
German cockroach	Greer	500 PNU/ml
Flea	Greer	1/1000 w/v
Mixed moths	Greer	500 PNU/ml
Horse fly (Tabanus)	Greer	500 PNU/ml
Mosquito (Culicidae)	Greer	500 PNU/ml
Moulds		
Aspergillus spp.	Greer	1000 PNU/ml
Alternaria tenuis	Greer	1000 PNU/ml
Botrytis cinerea	Greer	1000 PNU/ml
Penicillium spp.	Greer	1000 PNU/ml
Grain smut	Greer	1000 PNU/ml

Table 2.1 contd. Intradermal test allergens

Trees		
White ash	Greer	1000 PNU/ml
Ash	Artu	1000 NE/ml
Tag alder	Greer	1000 PNU/ml
Alder	Artu	1000 NE/ml
American Beech	Greer	1000 PNU/ml
Beech	Artu	1000 NE/ml
White oak	Greer	1000 PNU/ml
White birch	Greer	1000 PNU/ml
Birch	Artu	1000 NE/ml
American elm	Greer	1000 PNU/ml
Elm	Artu	1000 NE/ml
Box elder	Greer	1000 PNU/ml
Elder	Artu	1000 NE/ml
Eastern sycamore	Artu	1000 NE/ml
White pine	Greer	1000 PNU/ml
Weeds		
Yellow dock	Greer	1000 PNU/ml
Jerusalem oak	Greer	1000 PNU/ml
Lambs quarter	Greer	1000 PNU/ml
Common mugwort	Greer	1000 PNU/ml
Nettle	Greer	1000 PNU/ml
English plantain	Greer	1000 PNU/ml
Brassica spp.	Greer	1000 PNU/ml
Chrysanthemum spp.	Greer	1000 PNU/ml
Red clover	Greer	1000 PNU/ml
Grasses		
Kentucky blue grass	Greer	1000 PNU/ml
Meadow fescue	Greer	1000 PNU/ml
Orchard grass	Greer	1000 PNU/ml
Perennial rye grass	Greer	1000 PNU/ml
Sweet vernal grass	Greer	1000 PNU/ml
Timothy grass	Greer	1000 PNU/ml
Velvet / Yorkshire fog	Greer	1000 PNU/ml
Red top / bent grass	Greer	1000 PNU/ml
Couch grass	Greer	1000 PNU/ml

Table 2.2 Allergens tested in allergen-specific IgE serology

Timothy	Yellow dock	Aspergillus
Cocksfoot	Meadow fescue	Penicillium mix
Meadow grass	Beech	Cladosporum
Rye grass	Willow	Alternaria
Sheep sorrel	Poplar	*Acarus siro*
English plantain	Ash	*Tyrophagus putrescentiae*
Nettle	Maple (Sugar)	*Dermatophagoides farinae*
Lambs Quarter	Hazel	*Dermatophagoides pteronyssinus*
Mugworth	Pine (White)	Parakeet feathers
Alder	Grand fir	Cat epithelium
Oak	Plane tree	Cockroach
Birch (White)	Norway spruce	Flea saliva

2.2 Sample collection

2.2.1 Archived skin samples

Skin samples from atopic and healthy dogs previously collected in our laboratory by Dr. Tim Nuttall under Home Office licence (project licence no. 60:2336) subjected to the Animal (Scientific Procedures) Act 1986 were available for study. These samples were a very useful resource because they had been previously assayed using conventional RT-PCR and therefore allowed an opportunity for comparative study with newer methodologies.

Skin samples had been taken whilst the dogs were sedated for intradermal testing (Chapter 2.1.4.1) as follows. 0.5-1.0 ml 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. 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. The skin biopsies were taken with a 6-mm biopsy punch (Stiefel Laboratories, High Wycombe, UK) and placed immediately into RNAlater® (Ambion Inc., Huntingdon, UK) to preserve ribonucleic acid (RNA). Samples in RNAlater® were kept at 4°C overnight, and were transferred to a -20°C freezer for long-term storage. The biopsy sites were closed with a single interrupted polyglactin suture (Vicryl®; Ethicon Inc., Somerville, NJ, USA).

As some of these samples were a number of years old, the integrity of the RNA was checked prior to analysis (see Chapter 6.3.1).

2.2.2 Control tissue samples from healthy dogs

Skin biopsies were taken from healthy dogs presented for euthanasia immediately after confirmation of death. Popliteal lymph nodes were also excised from these

dogs to act as positive control material for the amplification of gene transcripts (Chapter 2.9). These tissues were divided into 5 mm^3 portions and stored in RNAlater® as described.

2.2.3 Serum collection

Archived atopic serum samples were available from two previous studies. The first was a study investigating antibody and cytokine responses in atopic dogs (Nuttall and others 2001b; Nuttall and others 2002a; Nuttall and others 2002b). The second was a study investigating the efficacy of allergen specific immunotherapy in atopic dogs (Colombo and others 2005). 10-20 ml blood samples had been collected into plain glass tubes by jugular venepuncture. Control blood samples were collected by cardiac puncture immediately after euthanasia. The tubes were kept at room temperature for 1-2 hours to allow clotting. After centrifugation at 2,200 g for 5 min, sera were obtained by collecting the supernatant, aliquoted and stored at -20°C until use.

2.3 House dust mite Dermatophagoides farinae extract

Whole body freeze-dried housedust mite *Dermatophagoide farinae* (*D. farinae*) antigen (Greer Laboratories, Lenior, NC, USA) was reconstructed into 1 mg/ml in sterile phosphate buffered saline (PBS), aliquoted and stored at -20°C until use.

2.4 Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis (SDS-PAGE)

2.4.1 Buffers and solutions

Resolving gel:

- 0.25 M Tris-HCl (Sigma, Dorset, UK) pH 8.8
- 10% w/v acrylamide/methylene bis-acrylamide solution (37.5:1) (ProtoGel®; National Diagnostics, Staffordshire, UK)

· 1% w/v sodium dodecyl sulphate (Fisher, Leicestershire, UK)

· 1% w/v ammonium persulphate (Bio-Rad Laboratories, Hempstead, UK)

· 0.1% v/v N,N,N',N'-tetramethyl-ethylenediamine (TEMED) (Sigma, Dorset, UK)

Stacking gel:

· 0.128 M Tris-HCl (Sigma, Dorset, UK) pH 6.8

· 4% w/v acrylamide/methylene bis-acrylamide solution (37.5:1) (ProtoGel®; National Diagnostics, Staffordshire, UK)

· 0.5% w/v sodium dodecyl sulphate (Fisher, Leicestershire, UK)

· 0.02% w/v ammonium persulphate (Bio-Rad, Hempstead, UK)

· 0.002% v/v N,N,N',N'-tetramethyl-ethylenediamine (TEMED) (Sigma, Dorset, UK)

Reducing loading buffer:

· 0.125 M Tris-HCl (Sigma, Dorset, UK) pH 6.8

· 4% w/v sodium dodecyl sulphate (Fisher, Leicestershire, UK)

· 20% v/v glycerol (BDH Chemicals Ltd., Poole, England)

· 0.005% w/v bromophenol blue (BDH, Poole, England)

· 10% v/v 2-mercaptoethanol (Sigma, Dorset, UK)

Non-reducing loading buffer:

· 0.125 M Tris-HCl (Sigma, Dorset, UK) pH 6.8

· 4% w/v sodium dodecyl sulphate (Fisher, Leicestershire, UK)

· 20% v/v glycerol (BDH, Poole, England)

· 005% w/v bromophenol blue (BDH, Poole, England)

Electrophoresis buffer:

· 25 mM Tris-HCl (Sigma, Dorset, UK) pH 8.3

· 0.2 M glycine (Fisher, Leicestershire, UK)

· 1.0% w/v sodium dodecyl sulphate (Fisher, Leicestershire, UK)

Molecular weight markers:

· Broad range protein marker (Bio-Rad, Hempstead, UK)

Coomassie blue:

· 0.1% w/v Coomassie Brilliant Blue R-250 (BDH, Poole, England)

· 25% v/v methanol (Scientific Laboratory Supplies, Nottingham, UK)

· 5% v/v acetic acid (Fisher, Leicestershire, UK)

Destain solution:

· 45% (v/v) methanol (Scientific Laboratory Supplies, Nottingham, UK)

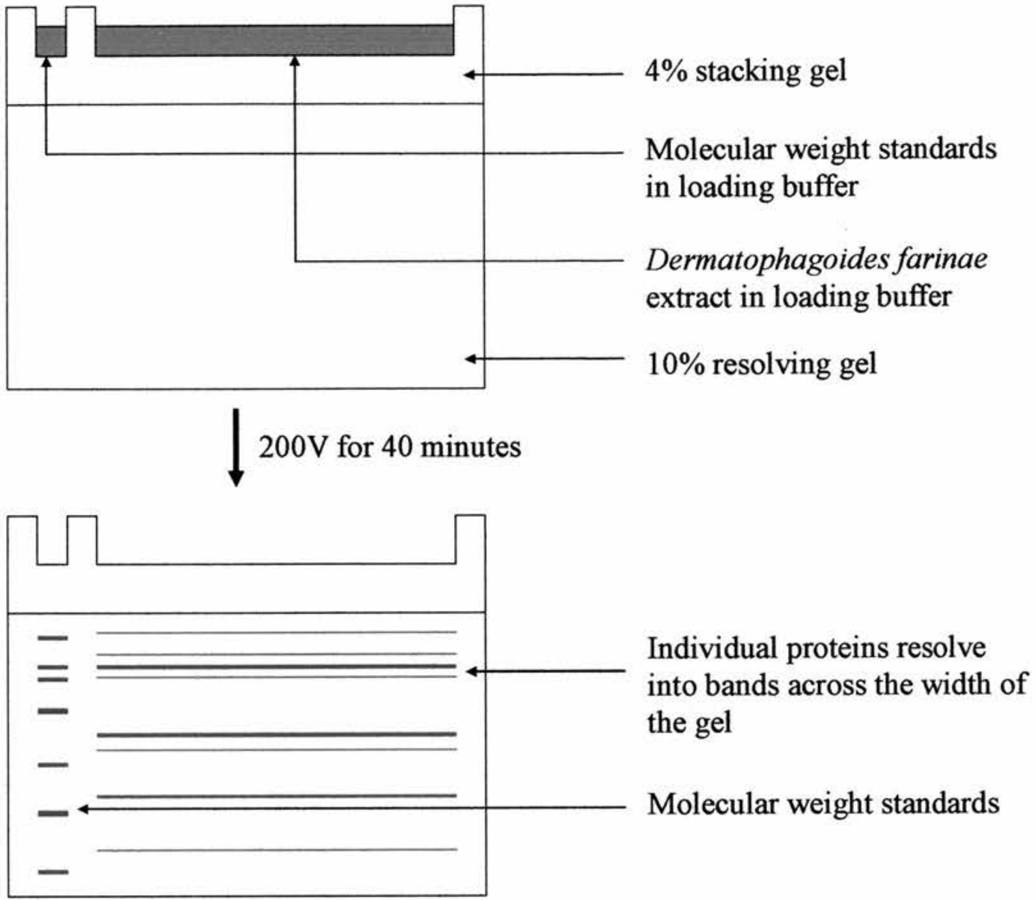
· 9% (v/v) acetic acid (Fisher, Leicestershire, UK)

2.4.2 Technique

The gel apparatus was assembled according to the manufacturer's instructions (MiniPROTEAN II®, Bio-Rad, Hempstead, UK). The glass plates were cleaned with 70% ethanol and SDS-PAGE was performed according to Laemmli's method (Laemmli 1970) using a 10% tris-glycine polyacrylamide resolving gel and a 4% stacking gel in a discontinuous buffer system. Ingredients for stacking gel and resolving gel were mixed in the order of acrylamide stock, water, Tris-HCl buffer, 10% SDS, ammonium persulphate and TEMED. Air bubble formation was avoided by gentle pipetting. Resolving gel buffer was carefully poured into the glass plate assembly and overlaid with water to ensure a flat surface and to exclude air as oxygen inhibits the polymerisation of the acrylamide. Water was drained off from the solidified resolving gel. Stacking gel buffer was poured on top of the set resolving gel, an appropriate comb was inserted to form the wells and allowed to set.

D. farinae extract and molecular weight standards were diluted 1:1 and 1:20 respectively with sample loading buffer and heated at 95°C for 5 minutes using a water bath. The extract was then layered into a single broad well with the molecular weight marker alongside (Figure 2.1). The gels were then placed in the electrophoresis tank according to the manufacturer's instructions (MiniPROTEAN II®, Bio-Rad, Hempstead, UK), filling the inner compartment with the tank buffer. Enough tank buffer was added to the lower compartment to cover the base of each gel. Electrophoresis was carried out at 200 V for 40 minutes, or until the dye front was within 2 mm of the bottom of the gel (Figure 2.1).

Figure 2.1 Schematic representation of a single well gel



2.4.3 Staining and imaging

2.4.3.1 Coomassie blue

Gels were stained in 0.25% Coomassie blue (BDH, Poole, England) for 10 minutes with gentle shaking. Background staining was eliminated by rinsing the gel in destain with frequent changes of solution.

2.4.3.2 Imaging of stained gels

Coomassie blue stained gels were transilluminated with white light and photographed (Kodak Digital Science™ Image Station 440CF, Kodak, Rochester, NY, USA).

2.5 Immunoblotting

2.5.1 Buffers and solutions

Tris-buffered saline (TBS):

- 20 mM Tris-HCl (Sigma, Dorset, UK) pH 7.5
- 0.5 M NaCl (Fisher, Leicestershire, UK)

Anode buffer I:

- 0.3 M Tris-HCl (Sigma, Dorset, UK) pH 10.4
- 10% v/v methanol (Scientific Laboratory Supplies, Nottingham, UK)

Anode buffer II:

- 25 mM Tris-HCl (Sigma, Dorset, UK) pH 10.4
- 10% v/v methanol (Scientific Laboratory Supplies, Nottingham, UK)

Cathode buffer:

- 25 mM Tris-HCl (Sigma, Dorset, UK) pH 9.4
- 40 mM 6-amino-n-caproic acid (Sigma, Dorset, UK)
- 10% v/v methanol (Scientific Laboratory Supplies, Nottingham, UK)

Blocking buffer:

- 5% skimmed milk (0% fat dried milk powder, Sainsbury, Edinburgh, UK) / TBS w/v

Diluting buffer:

· 1% skimmed milk (0% fat dried milk powder, Sainsbury, Edinburgh, UK) / TBS w/v

Washing buffer:

· 0.05% v/v Tween 20 (Fisher Leicestershire, UK) / TBS (TBS-T)

Immunological reagents

- Purified dog IgG (Sigma, Dorset, UK)
- Horseradish peroxidase (HRP) -conjugated polyclonal goat anti-dog IgG (Bethyl Laboratories Inc., Montgomery, TX, USA)
- Monoclonal mouse anti-dog IgE antibody (E6-71A1; Custom Monoclonals International, W. Sacramento, CA, USA)
- Monoclonal mouse anti-dog IgG subclass antibodies (mAbs B6, E5, A3G4 and A5; kindly provided by Dr. M. Day, University of Bristol, Langford, UK)
- HRP-conjugated bovine monoclonal anti-mouse IgG (MCA1421; Serotec, Kidlington, UK)
- 3,3'-diaminobenzidine (DAB) substrate (Vector Laboratories Inc., Peterborough, UK)
- Luminol (ECL™ (Amersham Pharmacia Biotech, Little Chalfont, UK)

2.5.2 Technique

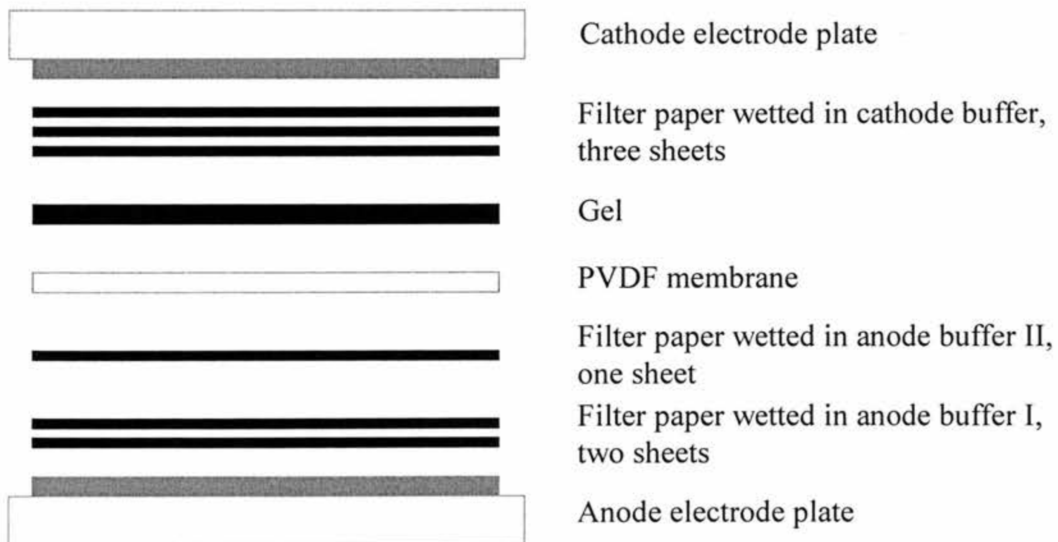
2.5.2.1 Electrophoretic transfer

Appropriate amounts of 1 mg/ml crude *D. farinae* extract were diluted in reducing or non-reducing sample buffer and 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.

Separated proteins on gels were transferred to 0.45μm polyvinylidene difluoride (PVDF) microporous membranes (Minipore Immunobilon™-P Transfer Membrane, Millipore Corporation, Bedfore, MA, USA). Gels were carefully removed from the glass cassettes and immersed in cathode buffer for 15 minutes to equilibrate. The PVDF membranes were pre-soaked in methanol for 15 seconds, and then rinsed in

anode buffer II for 5 minutes prior to blotting. For each transfer, two pieces of filter paper (Chromatography paper 3mm, Whatman International, Maidstone, UK) soaked in the anode buffer I and one piece soaked in the anode buffer II were laid on the anode electrode plate of the semi-dry electrophoretic transfer cell (Trans-Blot® SD, Bio-Rad, Hempstead, UK). Air bubbles between layers were removed by carefully rolling a pipette over the surface of each layer when assembling the transfer stack to ensure an even transfer. The PVDF membrane was placed on top of the filter papers and the unstained gel placed on top of the membrane. Another three sheets of filter papers soaked in cathode buffer were then placed on top of the gel. The transfer stack was assembled (Figure 2.2) and run at 80 mA per gel for 1 hour. After blotting, the apparatus was dismantled and one gel was stained with Coomassie blue (BDH, Poole, England) (Chapter 2.4.3.1) to ensure protein transfer had occurred. The PVDF membranes were air-dried and stored at 4°C.

Figure 2.2 Schematic representation of the Western blot transfer stack



2.5.2.2 Immunoblotting

The stored PVDF membranes were briefly immersed in methanol followed by equilibration 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 membranes were cut longitudinally into 4-mm wide strips (to facilitate testing of a large number of samples for antibodies directed against the blotted proteins) and placed into individual lanes of an 8-channel incubation tray (Bio-Rad, Hempstead, UK). All sites on the membrane which did not contain blotted protein from the gel were non-specifically blocked by immersing strips in 1 ml blocking reagent for one hour at room temperature on an orbital shaker, and washed with TBS-T three times for five minutes. Individual strips were incubated with predetermined, optimal dilution of serum samples (Chapter 3.2.5) for one hour at room temperature, followed by thorough washing with frequent changes of washing buffer. Secondary (and tertiary where appropriate) antibody was then added at appropriate dilutions and incubated at room temperature for different periods of time depending on the experiment.

2.5.3 Staining and imaging

The molecular weight standards were stained with Coomassie blue (BDH, Poole, England) (Chapter 2.4.3.1) as previously described. The substrate DAB was used for colorimetric blots and the luminol substrate ECL™ was used for chemiluminescent blots (See Chapters 3 and 4 for details). The DAB solution was prepared according to manufacturer's instructions. 1 ml of the working solution was added to each strip and incubated for one minute with gentle agitation, followed by washing with tap water. The strips were then air-dried and stored at room temperature. The chromogenic strips, along with the molecular weight standard, were aligned and digitally scanned using a flatbed scanner (Epson 1650, Hemel, Hempstead, UK) set at 8-bit grey scale. The scanned images were imported into the image analysis software (Chapter 2.5.4) and evaluated.

For chemiluminescent blots, the protein surface of the strips was covered with ECL for 1 minute. After draining off excess luminol solution, the strips were wrapped in cling film and placed protein side down on the lightproof image plate on a Kodak Digital Science™ Image Station 440CF Chemiluminescent Imaging System (Kodak, Rochester, NY, USA) along with Coomassie blue stained molecular weight markers, and recorded as digital image file using validated exposure conditions (Chapter 4.2). The Image Station 440CF (IS 440CF) is fitted with a high resolution charged coupled device (CCD) camera and is specifically designed for chemiluminescence and fluorescence imaging, but the system also provides chromogenic detection and general purpose laboratory imaging of autoradiography films, Coomassie Brilliant Blue and silver stained gels and cell culture plates. In addition, this highly sensitive, multi-purpose imaging system eliminates the complexities associated with darkrooms and central imaging labs as well as the high costs and potential hazards of radiation-based imaging systems.

2.5.4 Image analysis

The Kodak Digital Science™ 1D Image Analysis Software (Kodak, Rochester, NY, USA) was used to generate density profiles of bands on the gel and bands on the Western blot. This software can automatically search the defined lanes for bands and create a density profile for each lane. It identifies the bands that are present and generates a wide variety of quantitative data for each band, allowing quantitative measurements to be obtained without operator bias. The molecular weights of experimental bands were calculated by comparing their mobility relative to the molecular weight standards. The software could also determine the intensity of each band, which was proportional to the amount of protein present (see Chapter 3.2.5). The sum intensity was automatically calculated by addition of the intensity values for all the pixels identified within the rectangular area of the band. Net intensity was automatically calculated by addition of the intensity values for all the pixels identified within the rectangular area of the band followed by subtraction of the background intensity. Data generated for each band were exported to a spreadsheet for statistical analysis.

2.6 Molecular Cloning

2.6.1 Buffers, solutions and reagents

Tris-acetate-EDTA (TAE) buffer:

· 40 mM Tris-HCl (Sigma, Dorset, UK) pH 8.5

· 0.1% v/v acetic acid (Sigma, Dorset, UK)

· 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma, Dorset, UK)

Tris-acetate (TE) buffer:

· 10 mM Tris-HCl (Sigma, Dorset, UK) pH 7.5

· 1 mM EDTA (Sigma, Dorset, UK)

6× DNA electrophoresis loading buffer

· 15% w/v ficoll type 400

· 0.25% w/v bromophenol blue

· 0.25% w/v xylene cyanol

DNA electrophoresis loading buffer

· 0.25% w/v Orange G (Sigma, Dorset, UK)

Luria-Bertani (LB) broth

· 1% w/v tryptone (Difco, Surrey, UK)

· 0.5% w/v yeast extract (Gibco, Paisley, UK)

· 0.1% w/v NaCl (Sigma, Dorset, UK)

LB plate

· 1% w/v tryptone (Difco, Surrey, UK)

· 0.5% w/v yeast extract (Gibco, Paisley, UK)

· 0.1% w/v NaCl (Sigma, Dorset, UK)

· 1% w/v agar (Invitrogen, Paisley, UK)

2.6.2 DNA quantification

DNA was quantified by measuring its absorbance in a spectrophotometer (DU650 Spectrophotometer, Beckman-Coulter, USA) and optical density (OD) was measured at 260 and 280 nm. One OD_{260} unit was equivalent to 50 μg/ml double-stranded DNA (dsDNA). The concentration of DNA samples were calculated using the equation:

• **DNA μg/ml = OD_{260} of the DNA sample × 50 × dilution factor**

The purity of the samples was assessed by the ratio OD_{260}/OD_{280}. Samples with a ratio less than 1.8 indicated that contamination by protein might have occurred, and DNA samples were re-precipitated again.

2.6.3 DNA digestion with restriction endonucleases

Restriction enzymes *Eco*RI, *Nco*I and *Bcl*I were obtained from New England Biolabs (New England Biolabs Inc. Hertfordshire, UK) and *Not*I was obtained from Promega (Promega, Southampton, UK) and were used with the supplied buffers according to the manufacturer's instructions. The reaction was set up in a total volume of 20-200 μl and incubated for 2 hours at a 37 °C temperature block (dri-block® DB-ZA, Techne, UK) (50°C for *Bcl*I). The reaction mix consisted of DNA to be digested, 10% of the appropriate 10× buffer, 0.1 μl/ml bovine serum albumin (BSA, Promega, UK), approximately 1 unit of restriction enzyme per μg of DNA and nuclease free water (Promega, UK) to the final volume. Enzymes were inactivated by incubation at 65°C for 20 minutes to avoid interference with subsequent enzymatic reactions, i.e. amplification.

2.6.4 Plasmid DNA analysis by electrophoresis

DNA was analysed by gel electrophoresis on agarose gels (Invitrogen, Paisley, UK). 1% agarose gels were made by adding 3 g of agarose (Invitrogen, UK) to 300 ml of TAE buffer and dissolving by heating to boiling point in a microwave. The mixture was allowed to cool to approximately 50°C before the addition of 0.5 μg/ml ethidium bromide (Sigma, Dorset, UK). The gel was poured into a flat bed mould into which combs had been inserted, and then allowed to set for at least 1 hour before use.

DNA samples were made up to a 10-20 μl volume with TAE, and mixed with 1/6 volume of 6x loading dye (15% w/v ficoll type 400, 0.25% w/v bromophenol blue and 0.25% w/v xylene cyanol) or 1/2 volume loading buffer (0.25% w/v Orange G, Sigma) in distilled water. Electrophoresis was carried out at 100V in a horizontal tank (Bio-Rad, Hempstead, UK) containing TAE buffer. The size of DNA bands

was estimated by comparison with DNA molecular weight standards (DNA ladder, New England BioLabs, Hertfordshire, UK). Bands were visualised using a short wave UV transilluminator (Syngene, Scientific Laboratory Supplies, Ltd., UK) and recorded.

2.6.5 Preparation of Luria-Bertani (LB) medium and plates

To prepare 1 litre of LB medium, 10 g tryptone (Difco, Surrey, UK), 5 g yeast extract (Gibco, Paisley, UK) and 10 g sodium chloride (Sigma, Dorset, UK) were dissolved in 950 ml distilled water. The pH of the solution was adjusted to 7.0 with NaOH and the final volume was brought up to 1 litre. The solution was then subjected to autoclaving on liquid cycle for 20 minutes at 15 pounds per square inch (psi). After the solution was cooled to 55°C, 100 μg/ml of ampicillin was added and stored at 4°C.

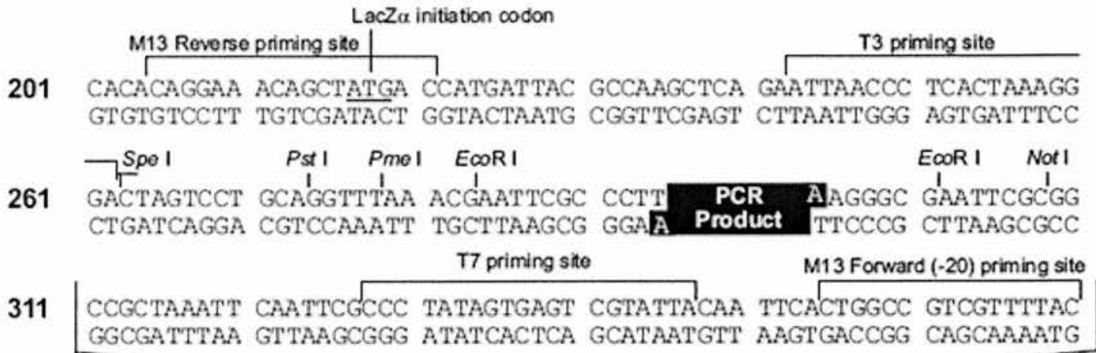
To prepare LB agar plates, LB medium was prepared as above and 10 g/l agar was added before autoclaving. After autoclaving and cooling to ~55°C, 100 μg/ml of ampicillin was added and the solution was poured into 10-cm plastic Petri dishes and allowed to solidify. Plates were then inverted and stored at 4°C in the dark.

2.6.6 Transformation of One Shot® Chemically Competent *Escherichia coli*

Fresh PCR products were cloned into a linear plasmid vector pCR®4-TOPO® (TOPO TA® Cloning Kit for Sequencing, Invitrogen, UK) (Figure 2.3). TOPO® Cloning reaction was set up using 0.5-4 μl PCR product, 1 μl salt solution and 1 μl TOPO® vector; sterile water was added to a final volume of 6 μl. The reaction was mixed gently and incubated for 5 minutes at room temperature and then placed on ice. 4 μl of the TOPO® Cloning reaction was added into a vial of One Shot® Chemically Competent *Escherichia coli*, mixed gently, and incubated on ice for 5 minutes. 50 μl of transformed cell suspension was placed on a pre-warmed LB plate (Chapter 2.7.5) and spread quickly and evenly over the plate using an L-shaped glass spreading tool and incubated overnight at 37°C. A small volume of the cell suspension was

streaked at one side of LB plate using a Nichrome loop. Streaking was repeated a few times (Figure 2.4) and the Nichrome loop was flamed and cooled down between each step. LB plates were placed with the agar facing downwards to minimize contamination and to reduce the chance of droplets of condensation falling on the agar surface.

Figure 2.3 pCR®4-TOPO® vector map



2.6.7 Small scale broth culture and isolation of plasmid DNA (Mini-prep)

A single bacterial colony picked from a freshly streaked selective plate using a flamed loop was inoculated into a culture of 1-5 ml LB medium (Chapter 2.7.5) and incubated for 12-16 hours at 37°C with vigorous rotational shaking at 250 revolutions per minute (rpm) (Orbital Shaker, Forma Scientific, UK). Tubes with a volume of at least 4 times the volume of the culture providing sufficient aeration were used. Plasmid DNA was purified using QIAprep® Spin Miniprep Kit (QIAGEN, West Sussex, UK) using the alkaline lysis method according to the kit manufacturer's instruction (Figure 2.5). Bacterial cells were harvested by centrifugation at 6800 g in a table-top microcentrifuge (Sigma 1-13, Scientific Laboratory Supplies, UK) for 3 minutes at room temperature and the supernatant discarded. Pelleted bacterial cells were resuspended in 250 μl Buffer P1 (Table 2.3), homogenised and transferred to microcentrifuge tubes. 250 μl Buffer P2 was added and capped tubes were inverted several times until the solution became viscous and translucent. 350 μl Buffer N3 was then added and mixed immediately and thoroughly by inverting the tubes 4-6 times followed by centrifugation at 17900 g for 10 minutes. The supernatants were then transferred to the QIAprep spin column by decanting or pipetting, centrifuged for 30-60 seconds, and the flow-through discarded. 0.5 ml Buffer PB was then added to the columns and centrifuged for 30-60 seconds and the flow-through discarded. 0.75 ml Buffer PE was then added and centrifuged for 30-60 seconds and the flow-through discarded. Residual washing buffer was removed by additional centrifugation for 1 minute. QIAprep spin columns were then placed in clean 1.5 ml microcentrifuge tubes. DNA was eluted by adding 50 μl Buffer EB to the centre of each spin column, followed by incubation for 1 minute and centrifugation for 1 minute. The samples were stored at 4°C and analysed by restriction enzyme digestion.

Figure 2.4 Procedure for the production of single colonies by the streak plate technique

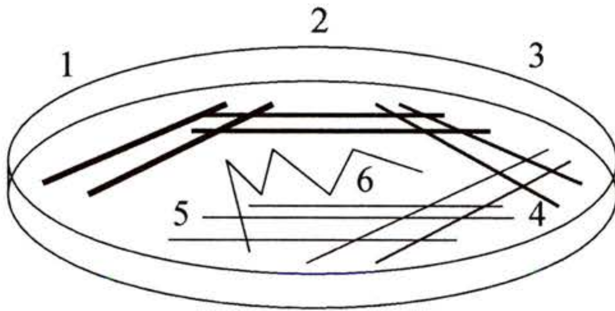


Figure 2.5 QIAprep spin procedure

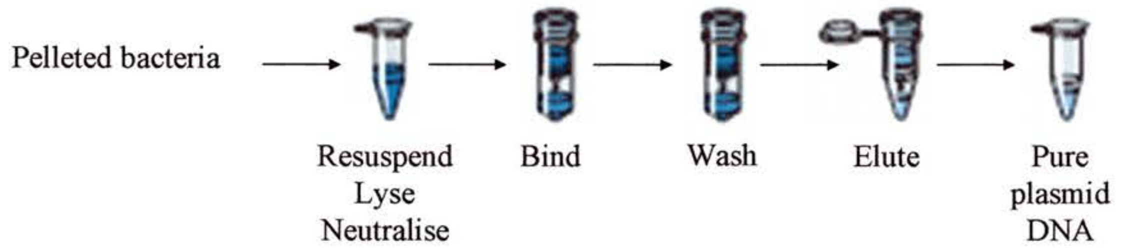


Table 2.3 Composition of buffers supplied in the plasmid purification kit

Buffer	Function	Composition
P1	Resuspension	50 mM Tris-HCl, pH 8.0 10 mM EDTA 100 μg/ml RNAseA
P2	Lysis buffer	200 mM NaOH 1% SDS (w/v)
N3	Neutralisation	(confidential)
EB	Elution	10 mM Tris-HCl, pH 8.5
P3	Neutralisation	3.0 M potassium acetate, pH 5.5
QBT	Equilibration	750 mM NaCl 50 mM MOPS, pH 7.0 15% isopropanol (v/v) 0.15% Triton®X-100 (v/v)
QC	Wash buffer	1.0 M NaCl 50 mM MOPS, pH 7.0 15% isopropanol (v/v)
QF	Elution	1.25 M NaCl 50 mM Tris-HCl, pH 8.5 15% isopropanol (v/v)

2.6.8 Large scale broth culture and plasmid DNA purification (Maxi-prep)

5-ml LB broth culture from a single colony was prepared (Chapter 2.6.7) and used as a starter material for the large-scale culture. The starter culture was diluted 1/500-1/1000 into selective LB broth and incubated overnight at 37°C on a rotary shaker (Forma Scientific, UK) at 250 rpm. Large-scale plasmid DNA was purified using QIAGEN® Plasmid Maxi kit (QIAGEN, UK) according to the manufacturer's instructions. An overnight bacterial culture (250 ml) was centrifuged at 6000 g for 15 minutes at 4°C (Model J2-21 Centrifuge, Beckman, UK) to harvest cells and the bacterial pellets were resuspended in 10 ml Buffer P1 (Table 2.3). 10 ml Buffer P2 was added and mixed thoroughly by vigorously inverting the sealed tube 4-6 times, and incubated at room temperature for 5 minutes. 10 ml of chilled Buffer P3 was then added and mixed immediately and thoroughly by vigorously inverting 4-6 times and incubating on ice for 20 minutes, followed by centrifugation at 20000 g for 30 minutes at 4°C and the supernatant collected promptly. The supernatant was subjected to centrifugation again at 20000 g for 15 minutes at 4°C, and the supernatant collected promptly. QIAGEN-tip 500 was equilibrated by applying 10 ml Buffer QBT and the column was allowed to empty by gravity flow. Previously collected supernatant was applied to the QIAGEN-tip and was allowed to enter the resin by gravity flow. The QIAGEN-tip was washed twice with 30 ml Buffer QC, and DNA was eluted with 15 ml Buffer QF.

2.6.9 Preparation of glycerol stock of transformants

For the preparation of glycerol stocks of bacterial cultures, 0.5 ml of sterile 60% glycerol was added to 1.5 ml of a log-phase bacterial culture. The culture was vortexed to ensure uniform dispersion of the glycerol before rapid freezing on dry-ice. After the culture was fully frozen, it was transferred to long-term storage at -70°C.

2.6.10 Automatic sequencing of DNA

DNA sequencing was performed using the di-deoxy chain termination sequencing method, by Mr. Ian Bennet (Department of Veterinary Pathology, University of Edinburgh). Reactions were performed using a Sequi-Therm EXCEL™ II DNA Sequencing kit LC on a 4000L automated sequencing machine (Li-COR DNA Sequencer, MWG-Biotech, Ebersberg, Germany).

2.6.11 Sequence analysis

Analysis of sequenced DNA by comparison to database sequences was done using the basic alignment search tool (BLAST) programs on the NCBI website http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul and others 1990).

2.7 RNA extraction and manipulation

2.7.1 RNA isolation

The homogeniser (Ultra-turrax 125®; Janke and Kunkel IKA, Staufen, Germany) probe was soaked overnight in dilute Decon Neutracon solution (Decon Laboratories, East Sussex, UK), washed thoroughly and subjected to several changes of 0.1% v/v diethylpyrocarbonate (DEPC)-treated water. The skin biopsy and positive control (lymph node) samples stored in RNA-later® (Ambion, Huntingdon, UK) were thawed and transferred into 50-ml Fulcan polypropylene phenol-resistant tubes (Fisher, Leicestershire, UK) containing 2.0 ml TRI-reagent® (Sigma, Dorset, UK). The samples were then homogenized on ice in 30-second bursts until fully dissociated in order to achieve optimal disruption of cells or tissues. Contents were transferred into 1.5 ml eppendorf tubes for the following procedure. 200 μl molecular biological grade chloroform (Sigma, Dorset, UK) was added and mixed vigorously by vortexing for 30 seconds in order to effectively denature the nucleoprotein complexes. After incubation for 5 min at room temperature, contents were centrifuged for 15 min at 9500 g at 4°C. The upper layer was collected into fresh tubes and precipitated with an equal volume of isopropanol (Sigma, Dorset, UK) at room temperature for 10 minutes. The tubes were then centrifuged at 3500 g for

10 minutes and the supernatant discarded. The RNA pellet was washed in 75% ethanol, centrifuged at 3500 g for 5 minutes and the supernatant again discarded. The washed RNA pellet was dissolved in 50-100 μl RNAse free water at 60°C for 10 minutes, and then chilled on ice.

2.7.2 DNAse treatment

Genomic DNA was removed from the RNA preparation by incubating with 4 units of recombinant DNase I (DNA-free®, Ambion, Huntingdon, UK) per 50 μl RNA solution at 37°C for 1 h. DNase I was removed by incubating with 0.1 volume of DNase Inactivation Reagent (DNA-free®, Ambion, Huntingdon, UK) at room temperature for 2 min with occasional mixing. After spinning at 3500 g for 1 min at 4°C, the supernatant was transferred into a new tube and chilled on ice.

2.7.3 RNA quantification

RNA was accurately quantified by measuring its absorbance in a spectrophotometer (DU650 spectrophotometer; Beckman-Coulter, Fullerton, CA, USA) and the optical density (OD) of RNA was measured at its maximum absorbance wavelength of 260 nm. One OD unit was equivalent to 40 μg/ml of RNA. The concentration of RNA samples were calculated using the equation:

- **RNA μg/ml = OD_{260} of the RNA sample × 40 × dilution factor**

The extraction process was repeated for samples with a 260/280 nm absorbance 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 until processed.

2.7.4 RNA analysis using Agilent Bioanalyzer

The electrodes of the bioanalyzer were cleaned with 350 μl of RNAse free water for 5 minutes to prevent contamination. Diluted ladder and gel-dye mixture were prepared according to manufacturer's instructions. Gel-dye mix, conditioning solution, marker, diluted ladder and samples were loaded into appropriate wells and

the chip was inserted in the Agilent 2100 Bioanalyzer (RNA 6000 Pico Assay, Agilent Technologies, UK) for analysis.

2.7.5 Reverse transcription of RNA

Approximately 1 μg of DNased RNA samples were reverse-transcribed in a final volume of 20 μl in a final concentration of 5 mM $MgCl_2$, 1× reverse transcription buffer (10 mM Tris-HCl [pH 9.0 at 25°C], 50 mM KCl, 0.1% Triton® X-100), 1 mM each dNTP, 1 U/μl recombinant RNasin® ribonuclease inhibitor, 15 U/μg avian myeloblastosis virus reverse transcriptase (AMV-RT) and 0.5 μg random primer (Reverse Transcriptase System, Promega, Southampton, UK) for 1 h at 42°C and 5 min at 99°C. The complementary DNA (cDNA) solution was then diluted to 100 μl in RNAse free water. cDNA preparations were stored at -20°C.

2.8 Polymerase Chain Reaction

2.8.1 Primer design

Internal oligonucleotide primers for amplifying canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH), interleukin-4 (IL-4), interferon-γ (IFN-γ), and transforming growth factor-β (TGF-β) had been previously validated in our laboratory by Dr. Tim Nuttall. Internal and external forward and reverse primers for canine inducible nitric oxide synthase (iNOS) and 18S ribosomal RNA (18S rRNA) along with external forward and reverse primers for canine IL-4, IFN-γ, TGF-β were designed from published canine mRNA sequences (GenBank®; National Centre for Biotechnology Information, URL – http://www.ncbi.nlh.nih.gov). The primer sequences were designed using an online primer design program Primer3 (Primer3, URL - http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to maximise specificity, priming efficiency and optimise annealing temperature, whilst minimizing secondary structure formation and complementarity to self or other primers. The primer sequences were screened against known sequences to eliminate any cross-reactive oligonucleotides (BLAST®; National Centre for Biotechnology Information; URL - http://www.ncbi.nlm.nih.gov/BLAST/). For real-time PCR,

primers were designed with optimal amplicon size of 100 bp. The oligonucleotide primers were generated by Operon Biotechnologies (Cologne, Germany) or MWG-Biotech (Ebersberg, Germany), diluted to 200 μM in nuclease-free water (Promega, Southampton, UK) and stored at -20°C.

2.8.2 Components of standard PCR reactions

Polymerase chain reactions (PCRs) were carried out in 50 μl volumes containing 2 μl cDNA, 0.2 μM forward and reverse primers, 2.5 units *Taq* DNA polymerase (Roche, East Sussex, UK) in a reaction buffer composed of 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.2 mM each deoxyribonucleotide triphosphates (dNTPs). The reaction conditions consisted of initial denaturing at 94°C for 2 min, then cycles of denaturing at 94°C for 30 sec, primer annealing for 30 sec and elongation at 72°C for 30 sec, before a final elongation at 72°C for 10 min (GeneAmp® PCR system 2400; Perkin-Elmer, Cambridge, UK). The optimum annealing temperatures of each primer pair was established in annealing temperature gradient experiments. GAPDH or 18S rRNA was used as the positive control housekeeping gene. Negative control reactions contained distilled water instead of template. Positive and negative control preparations were run in every PCR reaction. Samples with no positive control signal or a positive reaction from a negative control preparation were discarded.

2.8.3 PCR analysis by electrophoresis

10 μl of each PCR reaction or diluted plasmid DNA was mixed with 2 μl of the 6× loading dye and placed in the appropriate well of a 1.2% w/v agarose gel (Invitrogen, Paisley, UK) in TAE buffer containing 0.5 μg/ml ethidium bromide (Sigma, Dorset, UK). 2.5 μl DNA ladder was mixed with 0.5 μl loading dye and placed in lane 1 of each gel. Gels were run for 45 min at 100 V according to the manufacturer's instructions (Horizon II® gel system, Life Technologies). Completed gels were placed on the image plate on the Kodak Digital Science™ Image Station 440CF (Kodak, Rochester, NY, USA) and imaged under 590 nm ultra-violet light.

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

mRNA sequence	Accession number
GAPDH	AB038240
IL-4	AF083270
IFN-γ	AF126247
TGF-β	L34956
iNOS	AF077821
18S rRNA	AY262732

Table 2.5 Oligonucleotide primers for polymerase chain reactions

Cytokine	Primer sequence		Product size (bp)
GAPDH	Forward – CCTTCATTGACCTCAACTACAT Reverse – CCAAAGTTGTCATGGATGACC		400
IL-4	Inner	Forward – TAAAGGGTCTCACCTCCCAAACTG Reverse – TAGAACAGGTCTTGTTTGCCATGC	317
	Outer	Forward – GGCACGAGGTCTGCTATTGT Reverse – GCCTTTCCAAGAAGTCTTTCAG	403
	Real-time	Forward – TGATTCCAACTCTGGTCTGC Reverse – TTCTCGCTGTGAGGATGTTC	114
IFN-γ	Inner	Forward – TCGGACGGTGGGTCTCTTTTCG Reverse – CACTTTGATGAGTTCATTTATCGCC	281
	Outer	Forward – CGGCCTAACTCTCTGAAACG Reverse – TAAATGCACAACCCACAGGA	863
	Real-time	Forward – AAGCGGAAAAGGAGTCAGAA Reverse – CAAATATTGCAGGCAGGATG	75

Table 2.5 contd. Oligonucleotide primers for polymerase chain reactions

TGF-β	Inner	Forward – AGTTAAAAGCGGAGCAGCATGTGG Reverse – GATCCTTGCGGAAGTCAATGTAGAGC	434
	Outer	Forward – TCGACATGGAACTGGTGAAG Reverse – TAGTACACGATGGGCAGTGG	1000
	Real-time	Forward – ATTGACTTCCGCAAGGATCT Reverse – GTGTCCAGGCTCCAAATGTA	104
iNOS	Inner	Forward – TAGAGGAACATCTGGCCAGG Reverse – TTCCTCCAGGATGTTGTAGCG	706
	Outer	Forward – AGCCATTGAATTCGTCAACC Reverse – CAGGCCTCCACCTGGTAGTA	1062
	Real-time	Forward – GAAATCCCACCTGACCTTGT Reverse – CTCCCGGAACCACTCATACT	66
18S rRNA	Real-time	Forward – TGGTTGATCCTGCCAGTAGCA Reverse – ATGAGCCATTCGCAGTTTCACT	96

2.8.4 Real-time PCR analysis

Real-time PCRs were carried out in 20 μl volumes containing 1 μl cDNA, 100-1000 nM forward and reverse primers, 100 μM dNTPs, 1× reaction buffer, 1:28500 SYBR Green, 0.75 U *Taq* DNA polymerase, 0.4-3.2 mM of magnesium chloride and water to a final volume of 20 μl. Concentrations of the reagents used were determined after a series of dilution experiments (see Chapter 6). Appropriately mixed solutions for real-time PCR were loaded into 0.1-ml strip tubes (Corbett Research, Cambridge, UK) and placed on a 72-well rotor.

The reaction conditions consisted of initial denaturing at 94°C for 5 min, then cycles of denaturing at 94°C for 30 sec, primer annealing for 30 sec and elongation at 72°C for 30 sec, before a final incremental raise of temperature from 65°C to 94°C (Rotor-Gene™Real Time Thermal Cycler, Corbett Research, UK).

A standard curve for each cytokine was generated using cloned plasmid DNA. Negative control preparations were run in every real-time PCR reaction.

2.9 Statistical analysis

Graphical and descriptive statistical analysis of raw data was performed using Microsoft Office Excel 2003 (Microsoft Corp., Seattle, WA, USA), MINITAB™ Release 14 statistical software (Minitab Inc., PA, USA) and GraphPad Prism® 4 (GraphPad Inc., San Diego, CA, USA). Specific details of the tests used will be described in each experimental chapter.

Chapter 3

Dermatophagoides farinae-specific immunoglobulin G responses in healthy and atopic dogs

3.1 Introduction

The role of IgE in the pathogenesis of canine atopic dermatitis has been extensively studied, but the role of IgG is less well understood. In humans, IgG concentrations are elevated in various allergic conditions (Shakib and others 1977; Merrett and others 1984; Gondo and others 1987). Both total serum IgG or allergen-specific IgG can be raised in dogs suffering from AD, although dogs with no apparent clinical sensitivity to a particular allergen can also have elevated allergen-specific IgG (Willemse and others 1985b; Hill and others 1995; Day and others 1996; Fraser and others 2004). Additionally, allergen-specific IgG antibodies are markedly increased by allergen injection immunotherapy in humans (Ahlstedt and Eriksson 1977). IgG was reported to inhibit basophil histamine release and IgE-facilitated presentation of allergen by B cells to T-cell clones (van Neerven and others 1999; Wachholz and others 2003) in patients with pollen allergy receiving immunotherapy. Bee venom injection immunotherapy has been demonstrated to induce the formation of bee venom anti-idiotype antibodies (Khan and others 1991). Allergen-specific anti-idiotype antibodies could also be induced in other types of immunotherapy and bind to the antibody binding sites of specific IgE and thereby block the binding of allergen (Simon 2004). These observations have led to proposals that allergen-specific IgG antibodies might have protective properties in atopic individuals.

In the veterinary literature, non-IgE short term sensitising anaphylactic antibody of a subclass termed IgGd was demonstrated in dogs by the passive cutaneous anaphylaxis (PCA) test and Prausnitz-Küstner (PK) testing (Willemse and others 1985a). Unfortunately, determining that the results became negative after IgG absorption, which would have proven that the reaginic antibody was an IgG, was not done. The same group also showed that 89 % of the 62 atopic dogs studied had allergen-specific IgGd antibodies detected by ELISA although the agreement

between allergen-specific IgGd and intradermal test (IDT) ranged from 1/14 (7 %) for cat dander to 22/34 (65 %) for house dust (Willemse and others 1985b). In another study, IgGd to house dust antigens, *Dermatophagoides farinae* (DF) and *Dermatophagoides pteronyssinus* (DP) occurred with similar frequency in normal and atopic dogs, but significantly higher levels of DP-specific IgGd were found in normal dogs (Lian and Halliwell 1998). Furthermore, a high percentage of the atopic population had detectable IgGd to unrelated allergens, despite negative IDTs. The presence of IgGd directed against apparently irrelevant allergens in atopic patients and the high levels of IgGd in normal dogs to the most common allergens, DF and DP, implied an uncertain role of IgGd in canine atopic dermatitis (Lian and Halliwell 1998).

Nomenclature of the subclasses of canine IgG has changed over time and due to lack of exchange of reagents between researchers, establishing their precise identity is not possible. The functional equivalent of IgGd to the numerically classified canine IgG subclasses (IgG1, IgG2, IgG3 and IgG4, described further in Chapter 4) is not clear.

Hill et al (1995) showed that total IgG concentrations were significantly higher in atopic dogs compared to healthy controls (Hill and others 1995). Fraser et al (2004) has recently shown that concentrations of total IgG1 are higher in atopic dogs than in healthy dogs, suggesting that the atopic state also leads to activation of IgG subclass production in addition to IgE. A role for IgG has also been proposed in the mechanism of action of allergen-specific immunotherapy (ASIT) in dogs. Hites et al (1989) detected IgG antibodies with specificity for grass (June grass, Bermuda grass, rye grass, timothy grass and grass mix), weed pollen (careless weed, English plantain, giant ragweed, short ragweed and weed mix) and tree pollen (maple, olive, elm, birch and tree mix) allergens in the majority of non-atopic individuals and in all atopic subjects, and concentrations were highest in atopic dogs receiving allergen-specific immunotherapy (Hites and others 1989). Furthermore, Fraser et al (2004) has shown that concentrations of total IgG1 can increase following allergen-specific immunotherapy.

To date, compared to IgE, there is limited information on the specific IgG response to *D. farinae* antigens in atopic dogs. The aim of this study, therefore, was to develop and validate a semi-quantitative blot analysis system and use it to characterize and quantify the IgG response using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The immunoblotting technique was chosen because it provided a useful tool to show reactions between antibodies and various distinct allergens with known molecular weights. ELISA was not used in our study because immunoblotting allowed an overall surveillance of various allergen-specific antibody responses simultaneously, whereas in ELISA, specific responses could not be distinguished.

3.2 Validation and optimisation of methodology

3.2.1 Specificity of anti-canine IgG antibody

In order to characterise the IgG response to *Dermatophagoides farinae*, it was essential to demonstrate that the anti-canine IgG used in the study bound to IgG in dog sera specifically. The aim of the initial experiments was to validate the feasibility of using a horseradish peroxidase conjugated goat anti-dog IgG (Bethyl Laboratories, Montgomery, TX, USA) for immunoblotting.

3.2.1.1 Protein profiles of purified canine IgG and IgE

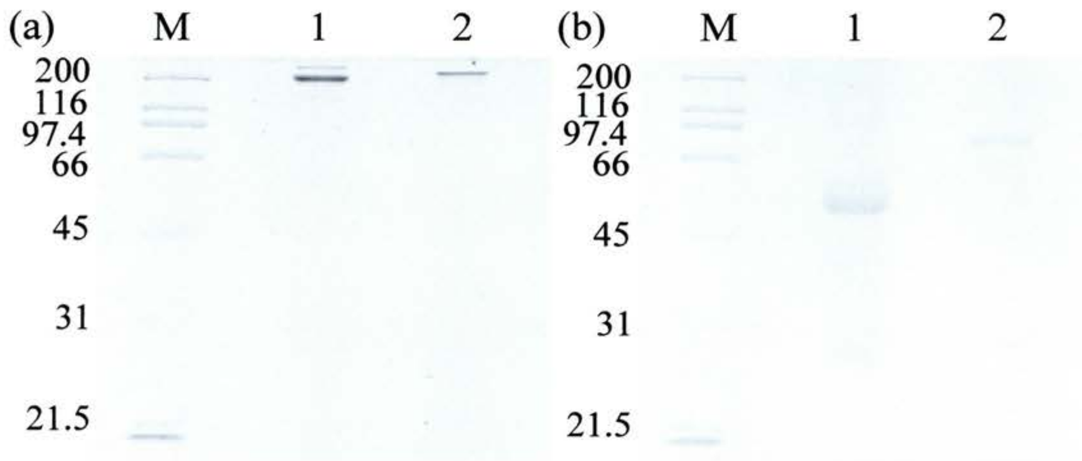
Prior to assessing the specificity of the anti-canine IgG and IgE, the purity of the canine IgG and canine IgE was examined by SDS-PAGE. 10 µl of 0.2 mg/ml purified canine IgG and IgE was diluted 1/1 in reducing and non-reducing sample buffer and separated by SDS-PAGE.

The protein profiles of purified canine IgG and IgE in SDS-PAGE gels are shown in Figure 3.1. Under non-reducing condition (Fig 3.1 a), intact IgG and IgE molecules had the molecular weight of approximately 190 and 200 kDa respectively. The purified canine IgG antibody was not composed of single protein band; a few bands around the 190 kDa protein were also present and might be different IgG subclass

molecules. Under reducing condition (Fig 3.1 b), antibodies were cleaved into heavy and light chains with molecular weights of approximately 54 and 24 kDa for IgG and 75 and 25 kDa for IgE.

Figure 3.1 Protein composition of the purified canine IgG and IgE used to assess antibody specificity in immunoblotting

Purified canine IgG (lane 1) and IgE (lane 2) analysed using non-reducing (a) and reducing (b) sample buffer in SDS-PAGE gels. M: molecular weight marker in kilodaltons (kDa).



3.2.1.2 Antibody specificity

In order to assess the specificity of the anti-canine IgG and IgE antibodies, 100 μl of 0.2 mg/ml purified canine IgG and IgE was diluted 1/1 in non-reducing sample buffer, separated by 10% SDS-PAGE gels using a broad well across the top (Chapter 2.4.2) and blotted onto PVDF membranes (Chapter 2.5.2), which were then cut into strips. The strips were probed with different combinations of the following antibodies diluted in dilution buffer: 1/1000 goat anti-canine IgG conjugated with horseradish peroxidase, 1/100 mouse anti-canine IgE, 1/1000 bovine anti-mouse IgG conjugated with horseradish peroxidase (Table 3.1). Immunoblots were developed using the chromogenic substrate DAB, or in the case of weak signals, the chemiluminescent substrate ECL, to visualise blotting results (Chapter 2.5.3).

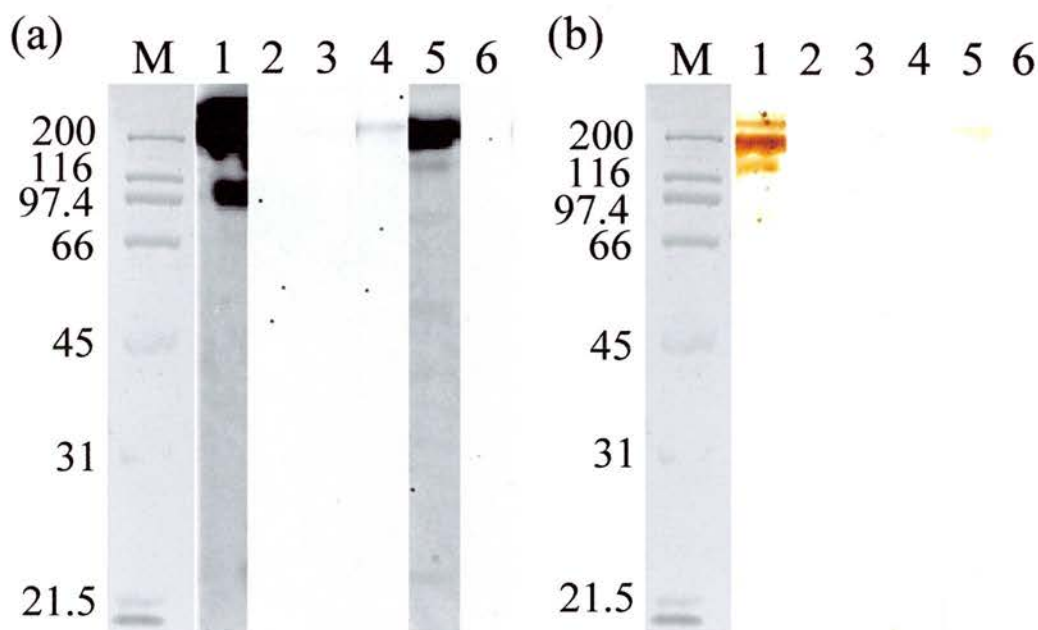
The binding of the anti-dog IgG and anti-dog IgE is shown in Figure 3.2 using chemiluminescent methods (a) and chromogenic methods (b). The signal in lane 1 represented the binding between goat anti-canine IgG to canine IgG antibody whereas the signal in lane 4 (visible in a) represented the binding between mouse anti-canine IgE to canine IgE. The results also demonstrated that goat anti-canine IgG could also bind to canine IgE, or that there was some IgG contamination of the IgE preparation (lane 5); however, the same quantities of IgG and IgE were used in this experiment whereas in physiological conditions, IgE concentrations would be a thousand fold less than that of IgG. Hence, when detecting canine IgG responses in serum using goat anti-dog IgG in immunoblots, the signal strength contributed from IgE antibody would be negligible.

Table 3.1 Antibodies used in immunoblotting assessing specificities

Strip	1	2	3	4	5	6
Ab on PVDF	IgG			IgE		
Primary Ab	Goat anti dog IgG-HRP	×	Mouse anti-dog IgE	Mouse anti-dog IgE	Goat anti dog IgG-HRP	×
Secondary Ab	×	Bovine anti-mouse IgG-HRP	Bovine anti-mouse IgG-HRP	Bovine anti-mouse IgG-HRP	×	Bovine anti-mouse IgG-HRP
Substrate	ECL					
Substrate	DAB					

Figure 3.2 Demonstration of binding between the horseradish peroxidase conjugated goat anti-canine IgG and purified canine IgG and IgE

Purified canine IgG (lanes 1-3) and IgE (lanes 4-6) were probed with goat anti-canine IgG, mouse anti-canine IgE as well as bovine anti-mouse IgG (Table 3.1) and visualised with luminol substrate ECL (a) and chromogenic substrate DAB (b). Goat anti-canine IgG bound to canine IgG (lane 1) and mouse anti-canine IgE bound to canine IgE (lane 4, not visible in b due to limited detection sensitivity of DAB). Goat anti-canine IgG also bound to canine IgE (lane 5). M: molecular weight standards in kDa.



3.2.2 Optimisation of *D. farinae* extract, canine serum and anti-canine IgG antibody dilution

In order to determine the optimal conditions to detect *D. farinae*-specific IgG responses in dog, reagents were tested at different dilutions. 50 µg and 100 µg *D. farinae* extracts (Greer, Lenior, NC, USA) were diluted 1/1 in reducing sample buffer, separated in two 10% SDS-PAGE gels and blotted onto PVDF membranes, which were then cut into strips. The strips were probed with a reference dog serum sample from an atopic dog known to be allergic to *D. farinae* (at 1/50, 1/100 and 1/200) and mouse anti-canine IgG conjugated with HRP (1/500, 1/1000 and 1/2000) (Table 3.2). DAB was incubated for one minute to visualise blotting signals.

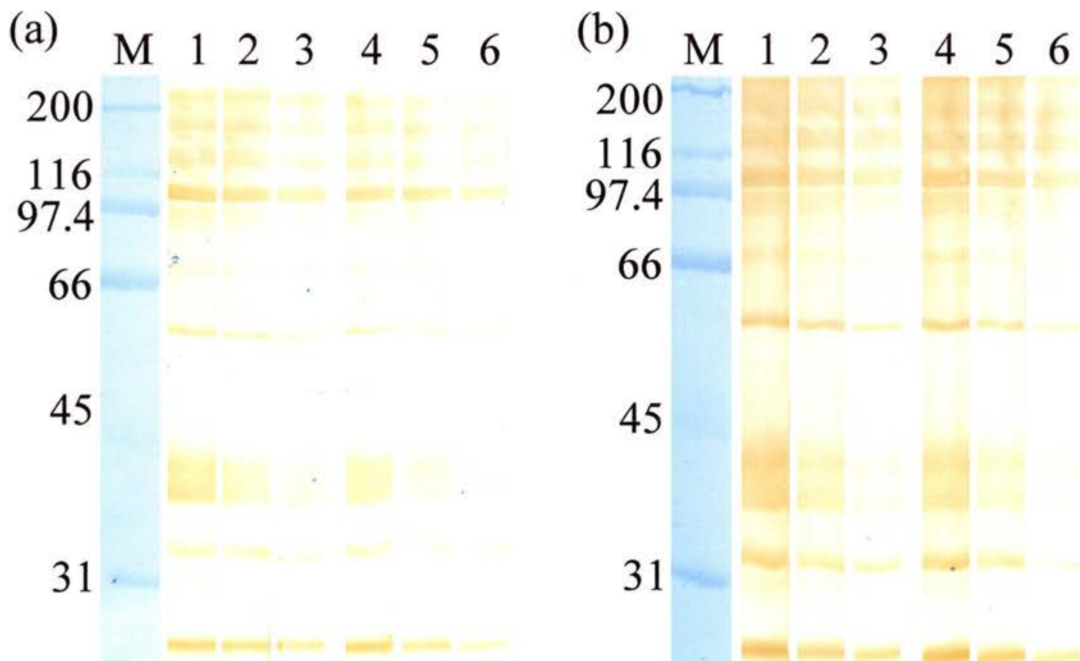
The results are shown in Figure 3.3 (a) (50 µg *D. farinae* extract) and Figure 3.3 (b) (100 µg *D. farinae* extract). Multiple antigens with molecular weights ranging from 20 kDa to 200 kDa were recognised by dog IgG antibody. As expected, the bands in Figure 3.3 (b) were generally stronger than those in Figure 3.3 (a) because twice the amount of *D. farinae* extract was present; however, the background staining was also stronger in Figure 3.3 (b). Also as expected, the general trend was that the less diluted the serum sample, the stronger the signals. The dilution of the anti-canine IgG did not have as much effect as the antigen quantity or serum dilution. Based on the results, the conditions chosen as being optimal in terms of signal strength/noise ratio were 50 µg *D. farinae* extract, serum at 1 in 100 and goat anti-canine IgG at 1 in 1000.

Table 3.2 Reagents used in immunoblotting assessing optimal conditions to detect *D. farinae*-specific IgG responses in dog

Blot	(a)						(b)					
Strip	1	2	3	4	5	6	1	2	3	4	5	6
D. farinae	50 μg						100 μg					
Serum	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{200}$
Anti-dog IgG-HRP	1/500			1/1000			1/1000			1/2000		
Substrate	DAB											

Figure 3.3 Optimisation of amount of *D. farinae* extract, dilution of dog serum sample and goat anti-canine IgG

50 μg (a) and 100 μg (b) *D. farinae* crude extract were separated in SDS-PAGE and transferred onto PVDF membranes for subsequent blotting. The reference serum sample was diluted 1/50 (lanes 1 and 4), 1/100 (lanes 2 and 5) and 1/200 (lanes 3 and 6) and goat anti-canine IgG conjugated with horseradish peroxidase diluted 1/500 (A: lanes 1-3), 1/1000 (A: lanes 4-6, B: lanes 1-3) and 1/2000 (B: lanes 4-6) (Table 3.2). The more diluted the reagents, the weaker the signals, the better the signal/noise ratio. M: molecular weight standards in kDa.



3.2.3 Optimisation of substrate incubation time

The chromogenic substrate DAB was chosen to visualise band signals and the incubation time was also critical because of potential saturation effects. The aim of this section was to evaluate the impact DAB incubation time had on band strength.

50 µg *D. farinae* extract diluted 1/1 in reducing sample buffer was separated in 10% SDS-PAGE gel and transferred onto a PVDF membrane. After removing the molecular weight standards, 16 4-mm wide strips were obtained and probed with 1/100 diluted dog serum sample and 1/1000 goat anti-canine IgG-HRP. Strips were then incubated with DAB for different periods of time ranging from 0 seconds to 20 minutes. Strips were dried overnight and scanned at a resolution of 200 pixels per inch (ppi) as digital files using a Hewlett-Packard Scanjet 3500c. Scanned files were then analysed using Kodak Digital Science™ 1D Image Analysis Software and data obtained by the software was imported into Microsoft Office Excel 2003 for analysis.

Immunoblotting strips incubated for different developing times are shown in Figure 3.4. Visible band strength and image analysis data increased with longer DAB incubation times. The signal strength of three prominent bands with MWs of 170, 130 and 98 kDa is plotted against DAB incubation time in Figure 3.5. Both net intensity and sum intensity results revealed the same trend which was the longer the DAB incubation time, the stronger the band strength. Band strength increased dramatically during the first 2 minutes and then gradually reached a plateau phase. One minute DAB incubation time was considered appropriate for future experiments as band intensity was not affected by colorimetric saturation and did not affect the semi-quantitative assessment of the IgG response.

Figure 3.4 Immunoblotting strips showing the relation between DAB incubation time and signal strength

16 strips subjected to the same blotting conditions were incubated with DAB substrate for different periods of time ranging from 20 minutes to 0 seconds. DAB incubation time for each strip is as follow: 1: 20 min; 2: 15 min; 3: 10 min; 4: 9 min; 5: 6 min; 6: 4 min; 7: 3 min; 8: 2min; 9: 90 sec; 10: 60 sec; 11: 50 sec; 12: 40 sec; 13: 30 sec; 14: 20 sec; 15: 10 sec; 16: 0 sec. M: molecular weight marker in kDa.

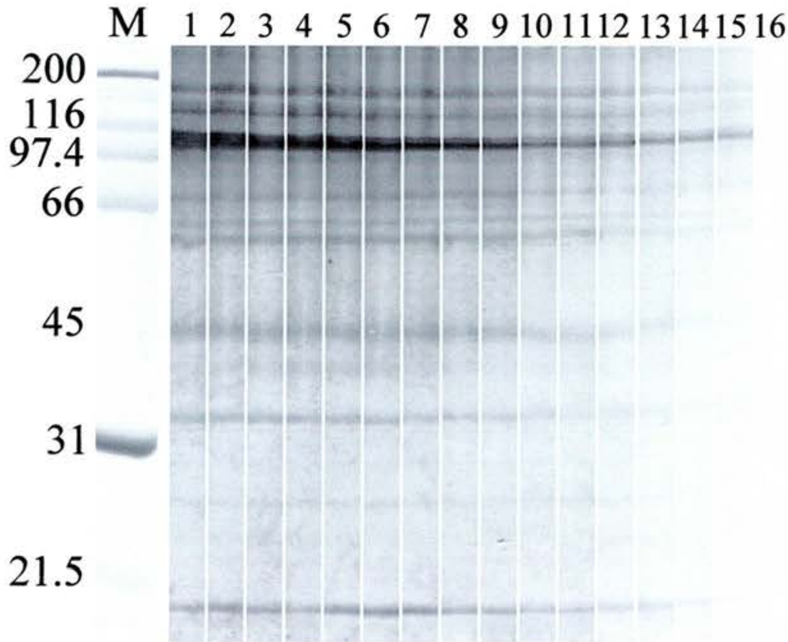
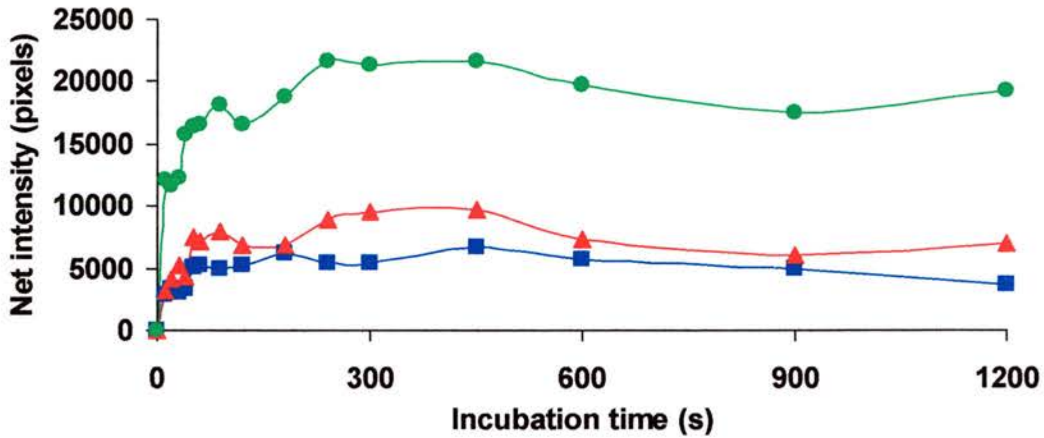
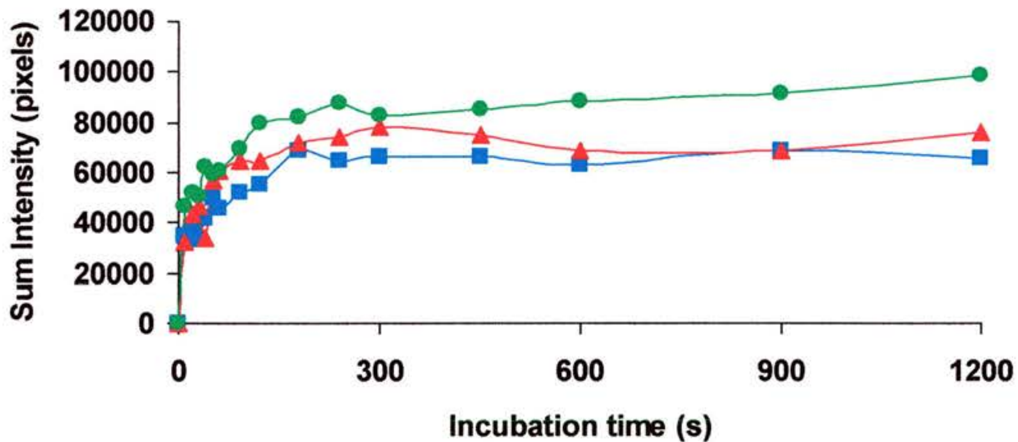


Figure 3.5 Demonstration of the relationship between DAB incubation time and signal strength

Blotting results were analysed using Kodak Digital Science™ 1D Image Analysis Software. Data from 3 representative bands with MWs of 170, 130 and 98 kDa are shown. Band net intensity (top) and sum intensity (bottom) plotted against DAB incubation time revealed the same trend: band intensity increased dramatically during the first 2 minutes and then reached a plateau phase afterwards. ■: 170 kDa antigen; ▲: 130 kDa antigen; ●: 98 kDa antigen.





3.2.4 Assessment of assay reproducibility

The purpose of this section was to evaluate the reproducibility of the immunoblotting conditions previously validated. 15 identical immunoblotting strips prepared as described in the previous section and subjected to the same blotting conditions are shown in Figure 3.6. The overall IgG binding profiles and the number of bands recognised were visually consistent between strips. Data from the image analysis software for 7 representative bands are summarised in Table 3.3. The coefficient of variation was calculated using the following equation:

- **$Cv = \sigma / \mu \times 100\ \%$**

(Cv: coefficient of variation; σ: standard deviation; μ: mean.)

The coefficient of variation ranged from 6 to 16 % for net intensity and 4 to 9 % for sum intensity. These intra-assay variation figures were considered to be acceptable for semi-quantitative analysis of band strength, as long as strips were only compared within the same experiment.

Figure 3.6 Immunoblotting strips showing assay reproducibility

15 strips containing separated *D. farinae* proteins were probed with the same canine serum, goat anti-mouse IgG-HRP, and visualised using DAB along with a negative control strip. The overall pattern was the same in all 15 strips. Band intensity as assessed by the image analysis software did not differ significantly. M: molecular weight marker in kDa.

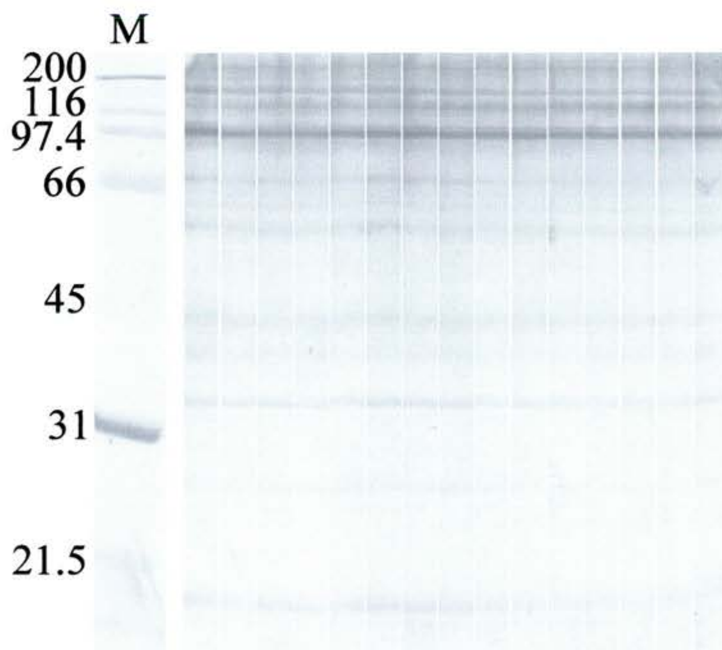


Table 3.3 Reproducibility of immunoblotting assay

	Net intensity			Sum intensity		
Band (kDa)	μ (pixels)	σ (pixels)	Cv (%)	μ (pixels)	σ (pixels)	Cv (%)
170	14716	1111	8	38039	3558	9
131	20529	1160	6	55185	4991	9
97	23766	1587	7	61703	2613	4
68	8643	1008	12	34633	2998	9
63	4578	717	16	25203	2392	9
44	5519	702	13	40316	1973	5
34	3942	601	15	37720	1845	5

Digital data showing net and sum intensities from 7 representative bands. The variation of band intensity measured by net intensity was slightly wider than that measured by sum intensity. μ: mean; σ: standard deviation; Cv: coefficient of variation.

3.2.5 Assessment of assay linearity

In order to further validate the immunoblotting and image analysis system for semi-quantitative measurements, it was important to evaluate assay linearity. The aim of this section was to determine whether changes in serum concentration resulted in a linear standard curve.

Twelve strips containing separated *D. farinae* proteins were probed with 3-fold serial dilutions of a standard serum sample from 1:10 to $1{:}10^6$. The standard serum sample was chosen because it gave a positive response to a number of protein bands in preliminary experiments. The blotting procedure was the same as previously validated and described.

Visual observation of the strips clearly showed that the more diluted the serum sample, the weaker the band signals were. *D. farinae*-specific IgG responses were not detectable in serum samples diluted more than 1000 fold. The most obvious bands with MWs of 170 kDa, 97 kDa, 66 kDa, 57 kDa, 44 kDa, 39 kDa, 34 kDa and 18 kDa were selected and analysed by the image analysis software. The results of the net intensity and sum intensity are shown in Figure 3.8 and Figure 3.9 respectively. The intensity of each band changed in a linear fashion between log serum dilution of 1/10 and 1/1000 and linear regression revealed R-squared values ranged from 0.9207 to 0.9917 for net intensity measurements and from 0.9370 to 0.9881 for sum intensity measurements. These results indicated that by using a serum dilution of 1/100, the system could be used to reliably semi-quantify IgG concentrations over a range that was 10× higher or 10× lower than those normally found in serum.

Figure 3.7 Effect of serum dilution on band strength

Separated *D. farinae* antigens transferred onto a PVDF membrane were probed with a reference canine serum sample at the following dilutions (from strip 1 to 11): 1×10^{-1}, 3×10^{-2}, 1×10^{-2}, 3×10^{-3}, 1×10^{-3}, 3×10^{-4}, 1×10^{-4}, 3×10^{-5}, 1×10^{-5}, 3×10^{-6} and 1×10^{-6}. No serum sample was added in strip 12 to serve as a negative control. Band signal intensity was reduced when serum concentration was decreased. When the serum sample was diluted to more than 1000 fold, *D. farinae*-specific IgG responses were not detectable.

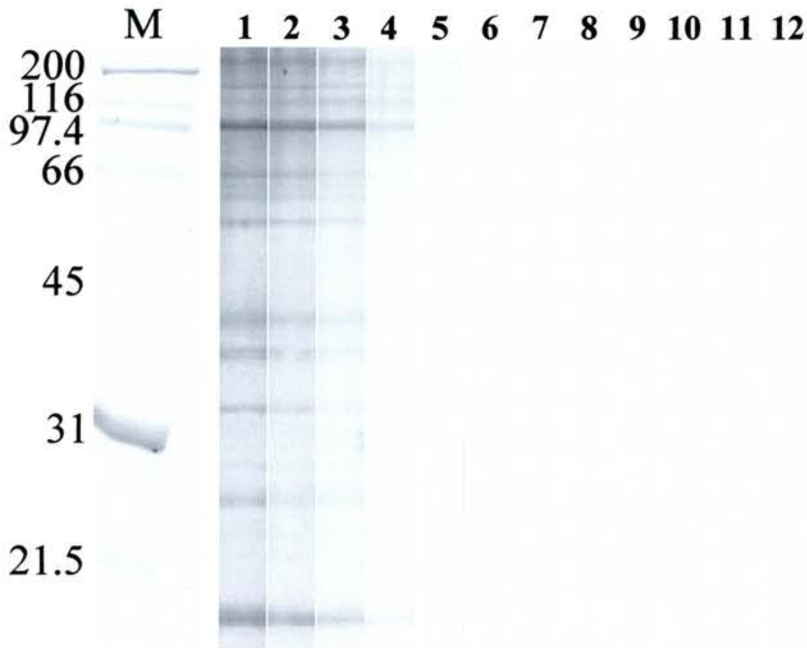


Figure 3.8 Demonstration of linear relationship between signal net intensity and serum concentration

Eight prominent bands with different MWs were chosen for digital image analysis. The serum concentration (µl/ml) is shown on the X-axis and net intensity (×10^4 pixels) is shown on the Y-axis. Regression lines and R-squared values are shown on each graph. These linear standard curves indicate that the image analysis system could determine changes in IgG concentration reliably enough to allow semi-quantitative analysis.

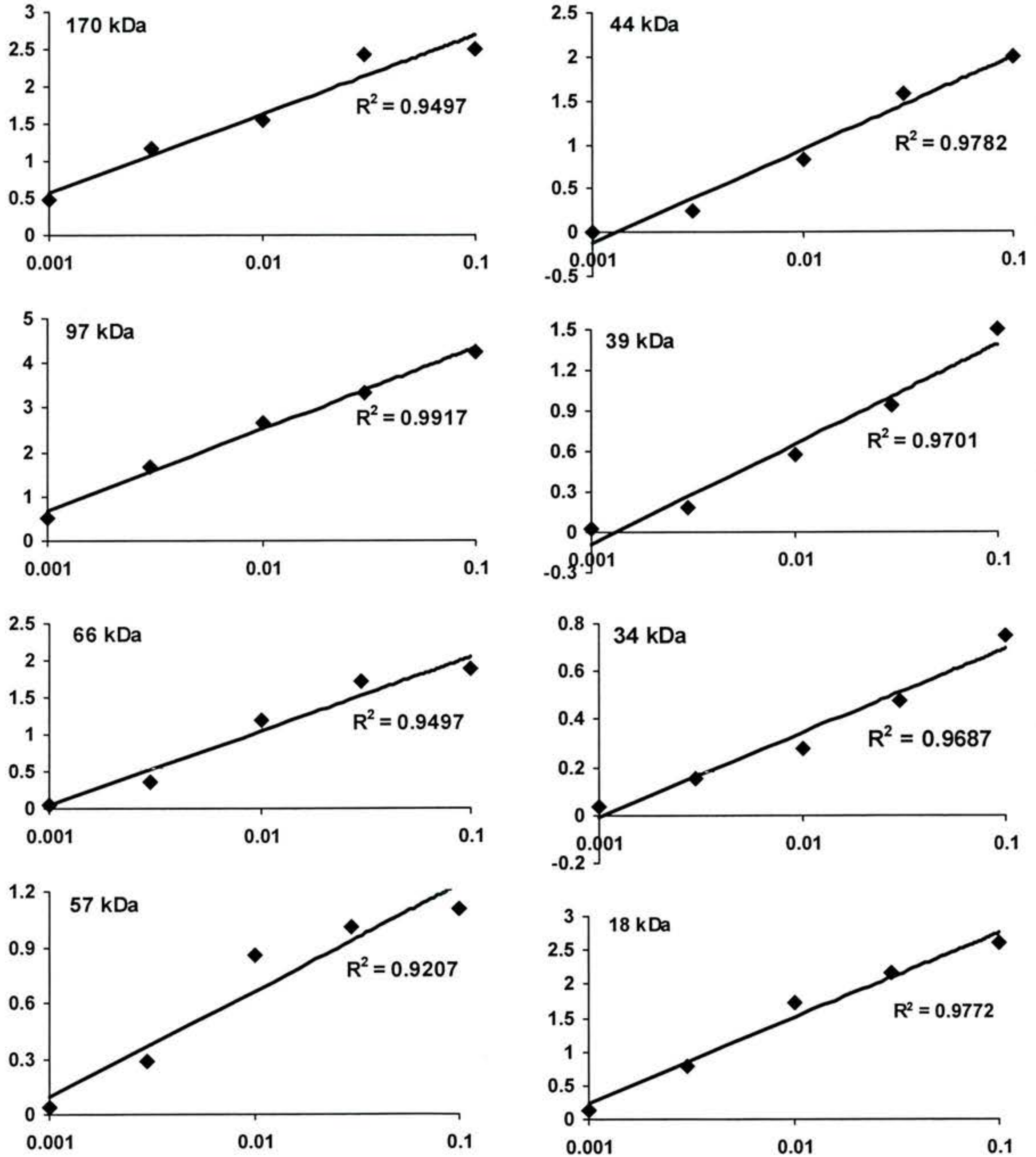
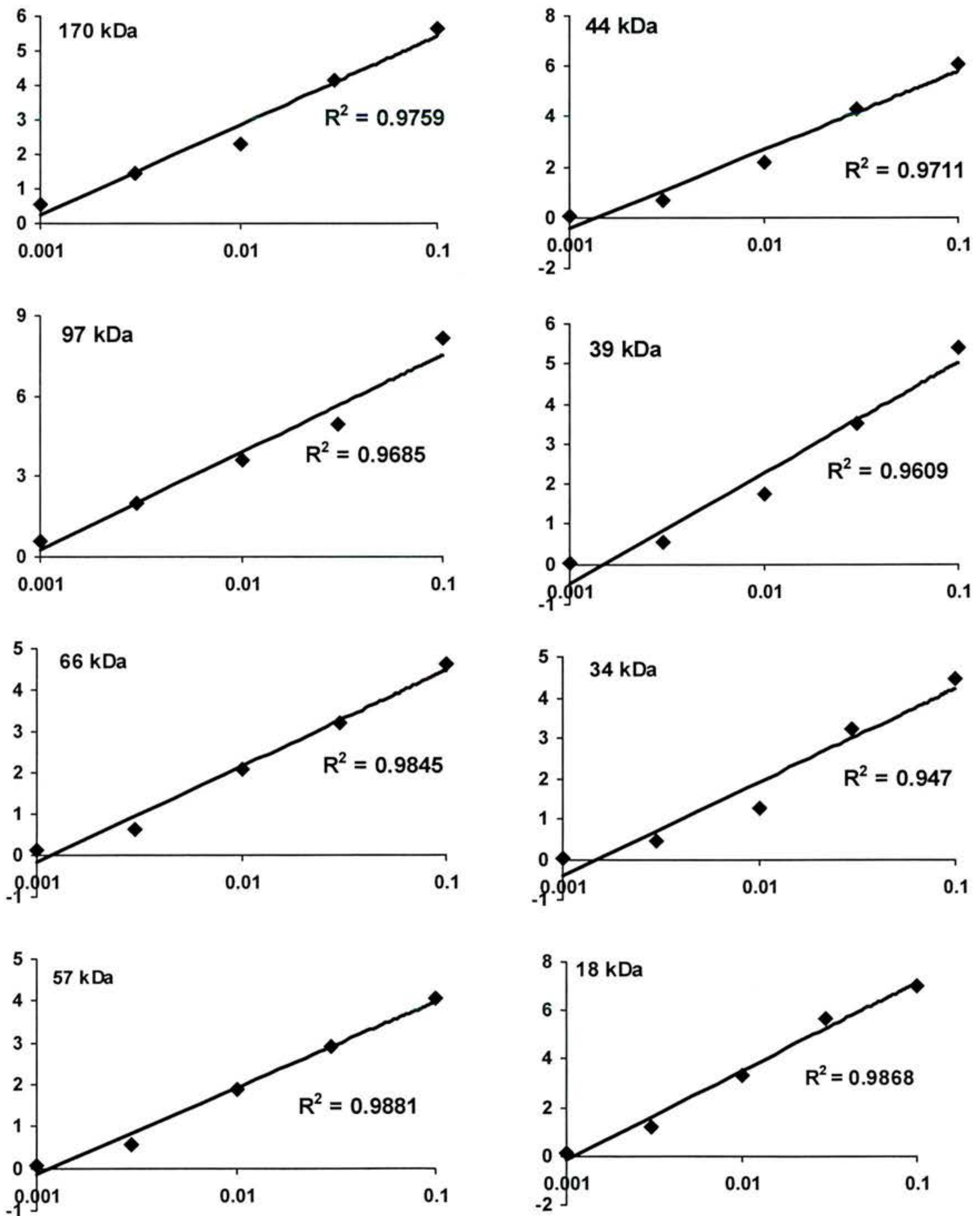


Figure 3.9 Demonstration of linear relationship between signal sum intensity and serum concentration

The serum concentration (μl/ml) is shown on the X-axis and net intensity ($\times10^4$ pixels) is shown on the Y-axis. Regression lines and R-squared values are shown on each graph.



3.3 Dermatophagoides farinae-specific IgG responses in healthy and atopic dogs

The objective of this section was to use the previously validated semi-quantitative blot analysis system to characterise and quantify *D. farinae*-specific IgG responses in a number of healthy and atopic dogs.

Serum samples collected from 20 healthy dogs (Chapter 2.1.2) and 20 atopic dogs (Chapter 2.1.3) were included in this study. 50 µg (1 mg/ml) *D. farinae* extract diluted 1/1 in reducing sample buffer was separated in 10% SDS-PAGE gel (Chapter 2.4.2) and electrophoretically transferred onto a PVDF membrane. The molecular weight standards were cut off and stained with Coomassie blue. The rest of the membrane was cut into sixteen 4-mm wide strips and blocked for 1 hour with 5% skimmed milk in TBS. In order to minimise inter-membrane variability, within each experiment, 8 strips were used to detect *D. farinae*-specific IgG responses from healthy dogs and the other 8 strips from the same membrane were used to detect IgG responses from atopic dogs. Assays were performed in duplicate. For immunoblotting, 1 ml of 1/100 diluted serum samples was added to individual strips for 1 hour at room temperature. After thorough washing, strips were incubated with 1 ml of 1/1000 diluted horseradish peroxidase conjugated goat anti-canine IgG for another hour at room temperature. After another round of thorough washing, individual strips were developed with 1 ml 3,3'-diaminobenzidine (DAB) peroxidase substrate for 1 minute and then removed by washing in distilled water. The strips were air-dried overnight prior to digital image acquisition and analysis.

The Mann-Whitney test was used to compare healthy and atopic dogs for the number of bands recognised, the molecular weight range of the bands observed, the total intensity of the bands in each group and the intensity of the most important individual bands. A p-value of <0.05 was considered to be significant.

A representative set of IgG immunoblots of individual dog sera from the two groups are shown in Figure 3.10. Both healthy and atopic dogs mounted an IgG response to multiple antigens from *D. farinae*. The two most visually obvious bands in both

groups were seen at molecular weights of approximately 98 and 44 kDa. The number of bands recognised did not differ significantly between the two groups (p = 0.6652; Figure 3.11) and varied between 3 and 12 in the healthy group and 2 and 10 in the atopic group, with median band numbers of 6.5 and 6, respectively. The percentage of dogs recognising antigens of various molecular weights is shown in Figure 3.12. In both healthy and atopic groups, two peaks of frequency were seen. The first occurred at around 100 kDa and predominantly comprised responses to the 98 kDa antigen. The second occurred between 30 and 60 kDa, a major proportion of which represented responses to the 44 kDa antigen. However, IgG responses were seen to multiple other bands ranging in molecular weights from 20 to 180 kDa (Figure 3.10 and Figure 3.12).

The intensity of each band, and therefore, the magnitude of the IgG response to that particular protein(s), was provided by the digital analysis software. The intensity of response in each dog to proteins of various molecular weights is shown in Figure 3.13. The most intense bands were seen at molecular weights clustered around 100 and 44 kDa. Hence, not only were these proteins the most commonly recognised (as seen in Figure 3.12), they also resulted in the greatest IgG response. The average of the total band intensity in each strip (i.e., for each dog) was 8335 in the healthy group and 9997 in the atopic group; the difference approached statistical significance (p = 0.0697). The total response to the 98 kDa proteins were similar in the atopic group (net intensity 18411) and healthy group (net intensity 16953) and was not significantly different (p = 0.4094). However, the total response to the 44 kDa antigen in the atopic group (net intensity 11088) was almost double that seen in the healthy group (net intensity 6410). Based on the standard curve for this protein, this actually represented a 6-fold increase in serum concentration, but the difference between groups was again not statistically significant (p =0.2689).

Figure 3.10 Binding of canine IgG to separated proteins of *D. farinae* in 20 healthy dogs (a) and 20 atopic dogs (b)

Each strip represented one dog. Similar IgG binding profiles were observed. The most visually obvious bands were 98 kDa and 44 kDa antigens. M: molecular weight standards in kDa.

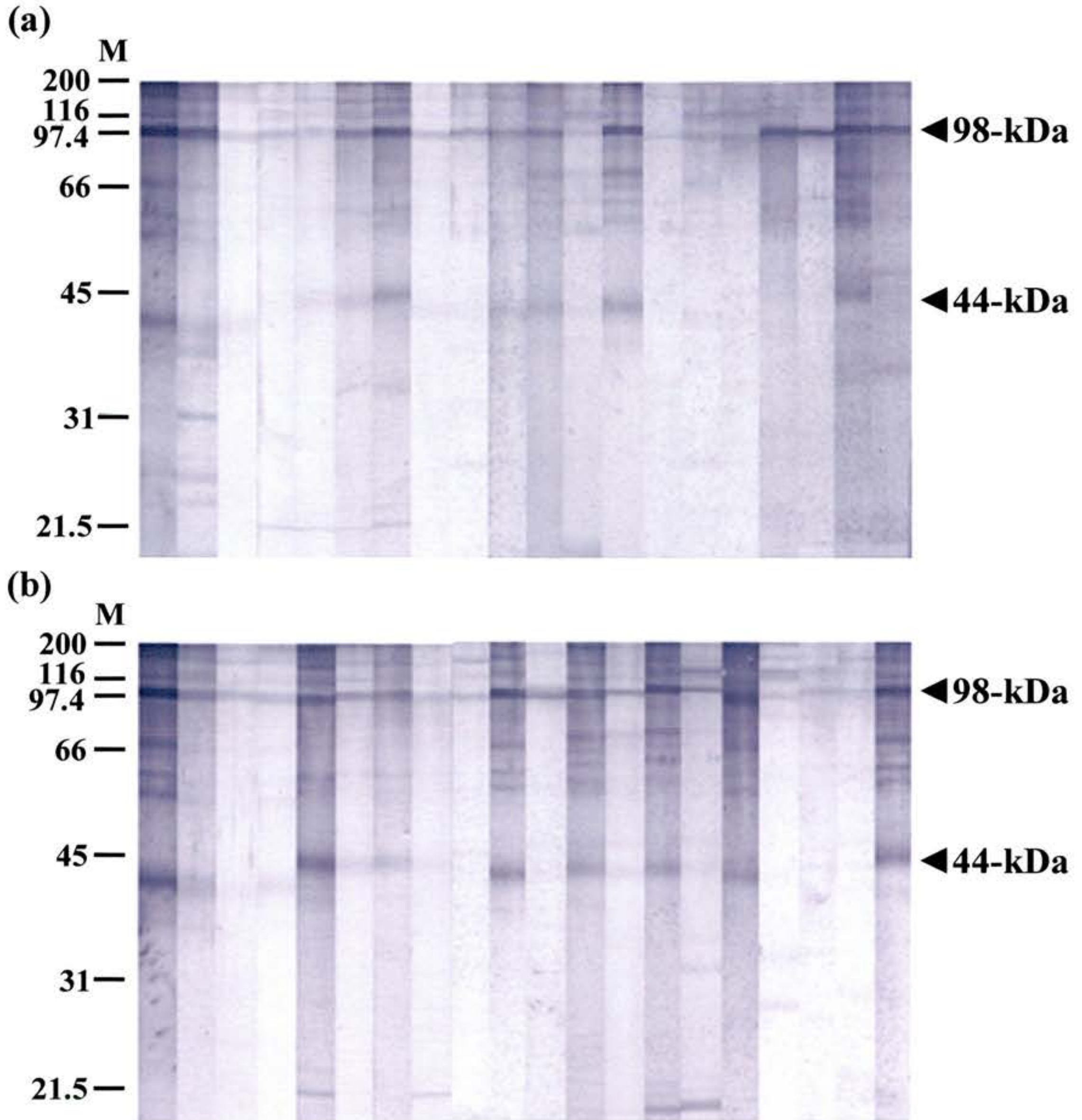


Figure 3.11 Number of *D. farinae* bands recognised by canine IgG in healthy and atopic dogs

Each box and whisker plot summarises the response of 20 dogs. The box represents the 25th and 75th quartiles with the horizontal bar indicating the median. The whiskers indicate the range.

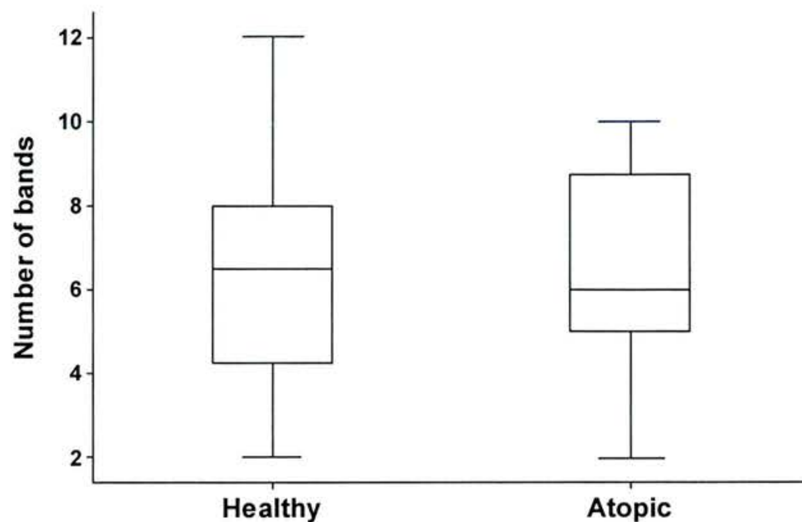


Figure 3.12 Proportion of healthy (a) and atopic (b) dogs recognising *D. farinae* antigens

Antigens are grouped in 10 kDa intervals and ranged from 15 to 185 kDa. A similar pattern was detected in both groups.

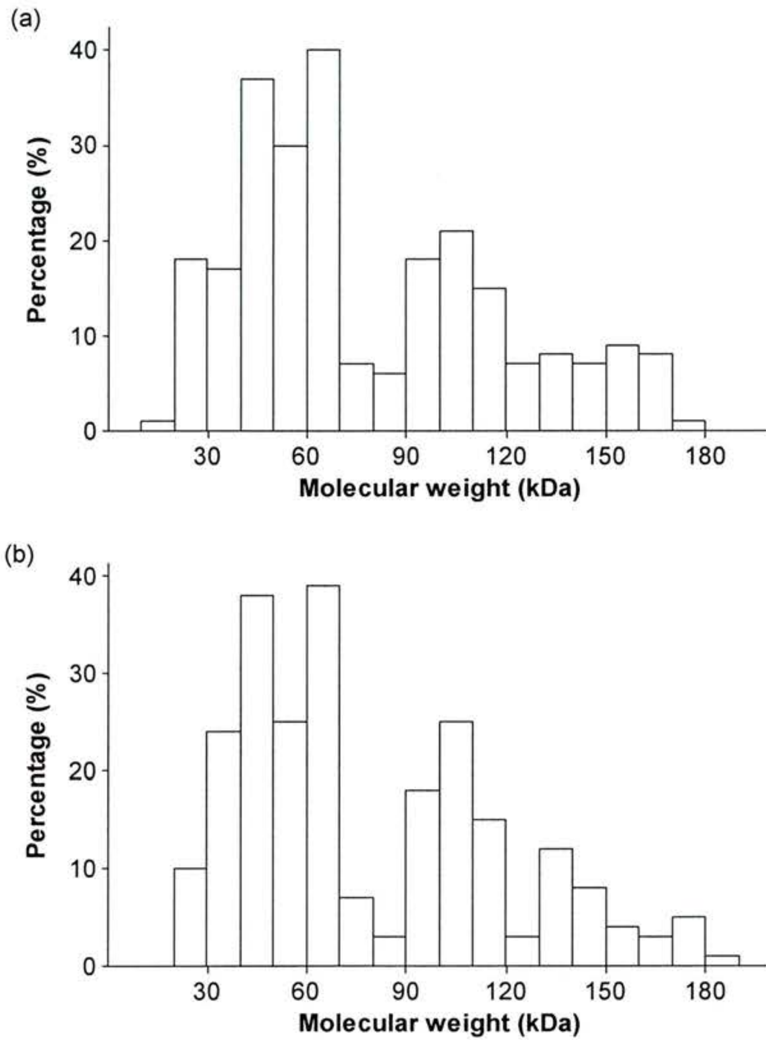
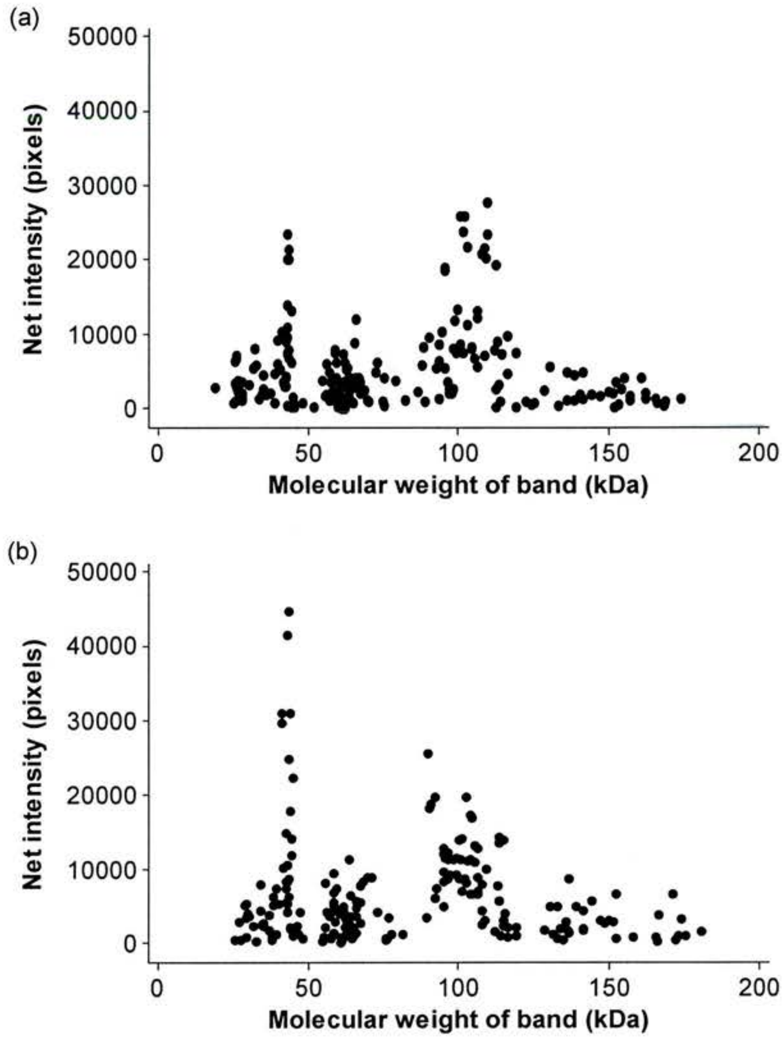


Figure 3.13 Scatter plot of band molecular weight against band net intensity in healthy (a) and atopic (b) dogs

Each dot represents an IgG response to a *D. farinae* protein in an individual dog. Note the two peaks of intensity around 100 and 44 kDa.



3.4 Discussion

In this chapter we aimed to develop and validate a semi-quantitative Western blot analysis system to allow us to investigate the IgG response to *D. farinae* allergens in healthy and atopic dogs. Initially, we demonstrated that polyclonal goat anti-canine IgG recognised dog IgG but it also cross-reacted with canine IgE in immunoblots using commercially available whole IgG and IgE preparations. However, the cross-reactivity of the reagent with canine IgE would not be relevant in these experiments because serum concentrations of allergen-specific IgE are so low that they cannot be detected using colorimetric blots, even when employing specific anti-canine IgE reagents. Interestingly, the anti-canine IgG appeared to have a higher affinity for IgE than the specific anti-IgE reagent, as evidenced by a stronger band on the blots.

The colorimetric saturation of substrates is of substantial importance if the data obtained is to be used for quantitative purposes. The development of the colour signal reached a plateau after approximately two minutes, beyond which there would be a loss of linearity. A 1 minute development time was therefore determined to be optimal to ensure that band intensity was not affected by colorimetric saturation.

The IgG response to *D. farinae* proteins allowed standard curves to be constructed with a linear and a logistic regression. The stronger the linear relationship between the two variables (serum dilution and band intensity), the higher the correlation and the more accurate the quantitative data that can be interpolated from the standard curve. Linearity of the data was assessed using the square of the correlation coefficient (R^2). The R^2 value for *D. farinae*-specific IgG versus signal net intensity and signal sum intensity ranged from 0.9207 to 0.9917 and 0.9470 to 0.9881 respectively (Fig. 3.8 and 3.9), indicating good linear fit over a quantifiable range of antibody concentrations. The intensity of each band changed in a linear fashion between log serum dilutions of 1/10 and 1/1000. By using serum dilutions of 1/100, the system could be used to reliably semi-quantify IgG concentrations over a range that was 10× higher or 10× lower than those normally found in serum.

Repeatability of the system was also assessed by probing 15 identical strips under the same blotting conditions and the intra-assay coefficients of variations of intensity for bands over the whole molecular weight range was typically around 10%. The inter-assay variation was not measured because all the samples were tested on the same day, atopic dogs were compared to healthy dogs within the same blot, and dilutions of reagents were made in batches. Inter-assay variation was therefore not considered to be relevant for these studies, but it would have to be carefully determined if the technique was used to investigate large numbers of dogs on different days.

Taken together, after determining the antibody specificity, assay reproducibility and linearity and confirming the absence of colorimetric saturation of signal intensity, the immunoblotting and image analysis system proved to be appropriate for semi-quantitative analysis of antibody concentrations. This system had a major advantage over ELISAs because it allowed antibody responses against separated antigens to be explored. If ELISAs had been used, it would only have been possible to determine the overall response to all the antigens present in the crude extract.

The image analysis system allowed *D. farinae*-specific IgG responses to be compared in healthy dogs and dogs with atopic dermatitis. The profile of IgG binding was similar in the two groups, both in terms of the number of bands recognised and their molecular weights. The percentage of individual dogs recognising these proteins was also similar in the two groups. These results indicate that *D. farinae* produces a number of proteins that are recognised by the canine immune system, both in healthy and atopic dogs.

The strength of the IgG response appeared to be greater overall, and for the 44 kDa band, in the atopic dogs than in the healthy controls. The lack of significant differences between the groups might imply that this difference was biologically irrelevant but this must be interpreted with caution. A larger number of dogs in each group would have increased the statistical power, which might have yielded a significant difference.

These results are in accordance with previous findings that demonstrated the presence of antigen-specific IgG antibodies in both healthy and atopic dogs using ELISAs (Willemse and others 1985b; Hites and others 1989; Day and others 1996; Lian and Halliwell 1998). Furthermore, the increased antigen-specific IgG response in the atopic group would be consistent with previous reports that showed higher concentrations of total IgG (Hill and others 1995) and total IgG1 (Fraser and others 2004) in atopic dogs. However, it must be borne in mind that these two previous studies measured total, and not antigen-specific, antibody concentrations. The total IgG responses seen in these studies could be directed at antigens other than *D. farinae* such as additional environmental allergens or proteins produced by *Malassezia* or staphylococcal organisms. Secondary infection with these pathogens is common in dogs with atopic dermatitis and previous studies have shown significantly greater IgG responses in atopic dogs to both Malassezia antigens (Nuttall and Halliwell 2001a; Chen and others 2002a) and staphylococcal proteins (Halliwell 1987; Morales and others 1994; Shearer and Day 1997).

The most important *D. farinae* antigens recognised, both in terms of frequency and magnitude were 98 and 44 kDa proteins. It has been previously shown that bands with the same molecular weights are recognised in IgE blots (Noli and others 1996; McCall and others 2001; Nuttall and Halliwell 2001a), although the 44 kDa protein is only seen when reducing buffers are used (Nuttall and Halliwell 2001a), suggesting that it might be a part of a bigger antigen. It is likely that the 98 kDa protein is the same as the high molecular weight chitinase that has recently been designated Der f 15 (McCall and others 2001). This protein is the most important allergen in atopic dogs that is recognised by IgE. However, the substantial IgG response to this protein indicates that distinct immune responses dominated by either isotype do not occur. Hence, although IgE responses are a critical component in the pathogenesis of canine atopic dermatitis, the concept of Th2 cytokine controlled class switching is clearly not an absolute phenomenon.

Taken together, the results of this and previous studies suggest that dogs mount an IgG response to multiple environmental antigens, including house dust mites. This

response is similar in healthy and atopic dogs, but is possibly greater in magnitude in atopic dogs. Hence, the presence of *D. farinae* antigens in the environment does not appear to be ignored by the canine immune system in healthy dogs and yet the IgG response does not appear to be protective against the development of clinical atopic disease. Whether the IgG response plays any direct role in the pathogenesis of the disease remains to be determined.

Chapter 4

Dermatophagoides farinae-specific immunoglobulin G subclass responses in normal and atopic dogs

4.1 Introduction

Although hypersensitivity is generally mediated by IgE reaginic antibodies, IgG anaphylactic antibodies have been demonstrated and characterised in a number of other species (Binaghi 1983; Goodwin 1983). The nonreaginic anaphylactic antibodies in different animal species share common features and differ from IgE antibodies in heat susceptibility, mercaptoethanol sensitivity and short-term sensitising (S-TS) property. IgG anaphylactic antibodies have been identified as IgG4 in man (Reid and others 1966) and guinea pigs (Margni and Hajos 1973), IgG1 in mice (Nussenzweig and Benacerraf 1964), IgG2a in rats (Bach and others 1971) and IgG2 in sheep (Esteves and others 1974). These antibodies are all relatively unaffected by heat or sulfydryl reduction and their persistence at passively prepared skin sites is much shorter than that of IgE. The demonstration of their anaphylactic activity in animals can be achieved by the passive cutaneous anaphylaxis (PCA) test, which is similar to the Prausnitz-Küstner reaction. In terms of biological activity, the existence of IgG anaphylactic antibodies in so many species must reflect a physiological need (Binaghi 1983). However, the role of IgG anaphylactic antibodies in atopic dermatitis was not investigated in these studies performed in the late 1970s and early 1980s.

The nomenclature to designate the subclasses of IgG in animals was mostly based on historical grounds and did not take into consideration the possible structural homologues between subclasses of different species. Although IgG antibodies and their associated effector systems were obviously conserved and homologous between species, the numbers of IgG subclasses vary and direct functional equivalence between subclasses in each species might not exist.

Four subclasses of IgG have been identified in humans and they received the denominations of IgG1, IgG2, IgG3 and IgG4 according to serum concentrations in normal subjects. Similar nomenclature was applied to canine IgG subclasses (Mazza and others 1993). Studies investigating the IgG subclasses in various allergic conditions in humans supported possible involvement of IgG subclass antibodies in allergic diseases.

High levels of IgE and IgG4 subclass were observed in the serum of AD patients, suggesting the involvement of IgG4 in some immediate hypersensitivity diseases (Shakib and others 1977). Specific IgG4 to environmental allergens was also detected and high levels of this antibody were found in patients with AD and allergic respiratory diseases (Nakagawa and others 1983; Merrett and others 1984). Walker first reported an association between the exacerbation of eczema in patients with AD and the exposure to airborne allergens such as pollen and house dust (Walker 1918). There have been many studies correlating worsening of symptoms and exposure to dust mites, as well as improvement of signs after environmental control (Beck and Korsgaard 1989; Harving and others 1990; Colloff 1992; Sanda and others 1992).

Studies investigating allergen-specific IgG subclass responses in various allergic conditions, however, demonstrated that specific subclass responses were allergen-dependent. Patients suffering from extrinsic allergic alveolitis (EAA) due to *Aspergillus fumigatus* typically demonstrated strong IgG subclass reactivities with a predominance of IgG1 and IgG2, while no specific IgE was found (Trompelt and others 1994). In allergic bronchopulmonary aspergillosis (ABPA) patients showed strong reactivities of all IgG subclasses and IgE but an intense IgG4 reaction appeared as a typical marker for ABPA (Trompelt and others 1994). It has also been shown in atopic children sensitised to birch pollen that specific antibodies of the IgG1 and IgG4 subclasses to the major birch-pollen allergen, Bet v1, are found in 93% of patients, whereas non-atopic children generally have no/low allergen specific IgG to birch pollen (Harfast and others 1998). Kemeny et al demonstrated that IgG1 antibodies to house dust mite (HDM) were detected in most atopic patients (94%) and healthy controls (97%), and an IgG4 response to HDM was present in 66%

atopics and 53% non-atopics (Kemeny and others 1989). However, the level of IgG4 antibodies were significantly higher in the atopic group (Kemeny and others 1989). Another study investigating specific IgG subclass responses in asthmatic patients sensitised to *D. farinae* has shown that the level of Der f1-specific IgG2 was significantly higher in the non-atopic controls than in the atopic group, but the Der f2-specific IgG2 and IgG4 were significantly higher in the atopic group than in the control group (Hong and others 1994). Mori et al has recently detected elevated levels of storage mite *Blomia tropicalis*-specific IgG3 subclass in AD patients and asthmatic patients (Mori and others 2001).

There are only a few studies of specific IgG subclasses to various allergens in dogs with atopic dermatitis. Studies by Day (Day and others 1996) suggested that IgG1 and IgG4 were involved in canine AD, depending on the allergen. Using a panel of monoclonal antibodies (mAbs) directed against the four subclasses of canine IgG (Mazza and others 1993; Mazza and others 1994b), it was demonstrated that the IgG response to *D. pteronyssinus* and *D. farinae* was dominated by the IgG4 subclass with significantly greater responders in atopic than normal dogs (Day and others 1996). In addition, significantly higher levels of IgG1, IgG2 and IgG3 antibodies were produced to *D. pteronyssinus* in the atopic than in control samples. However, the allergens involved could not be identified in the ELISA assays used.

The situation in dogs is further complicated by the description of IgGd (Willemse and others 1985a), a subclass that does not correspond with the 1-4 nomenclature (Day and others 1996). Experiments performed by Willemse et al in 1985 (Willemse and others 1985a; Willemse and others 1985b) demonstrated the presence of non-IgE anaphylactic antibodies, termed IgGd, in the sera of atopic dogs. It was suggested that this subclass could initiate immediate hypersensitivity. However, the role of IgGd in atopic diseases is uncertain; IgGd directed against apparently irrelevant allergens is present in atopic dogs, and there are high levels of IgGd in normal dogs to the common allergens *D. pteronyssinus* and *D. farinae* (Lian and Halliwell 1998). It is possible that IgGd corresponds to IgG1 (Richard Halliwell, personal communication) but this has not been proven. In contrast, antiserum specific for

IgGd failed to identify any of the four canine IgG subclasses in purified form in ELISAs (Day and others 1996). This may reflect the fact that IgGd is a myeloma protein, and the antiserum may not necessarily recognize native protein.

To date, nothing is known about the individual allergenic proteins targeted by IgG subclasses in dogs with AD because these have not been evaluated in previous ELISA assays using crude allergen extracts. Therefore, the aim of this study was to further develop and validate the semi-quantitative, digital, chemiluminescent western blot analysis system to evaluate allergen-specific IgG subclass responses to electrophoretically separated allergens of *Dermatophagoides farinae* in dogs with atopic dermatitis. A specific aim was to determine if different subclasses recognised specific proteins within the separated extract.

4.2 Validation and optimisation of methodology

4.2.1 Assessment of purified canine IgG

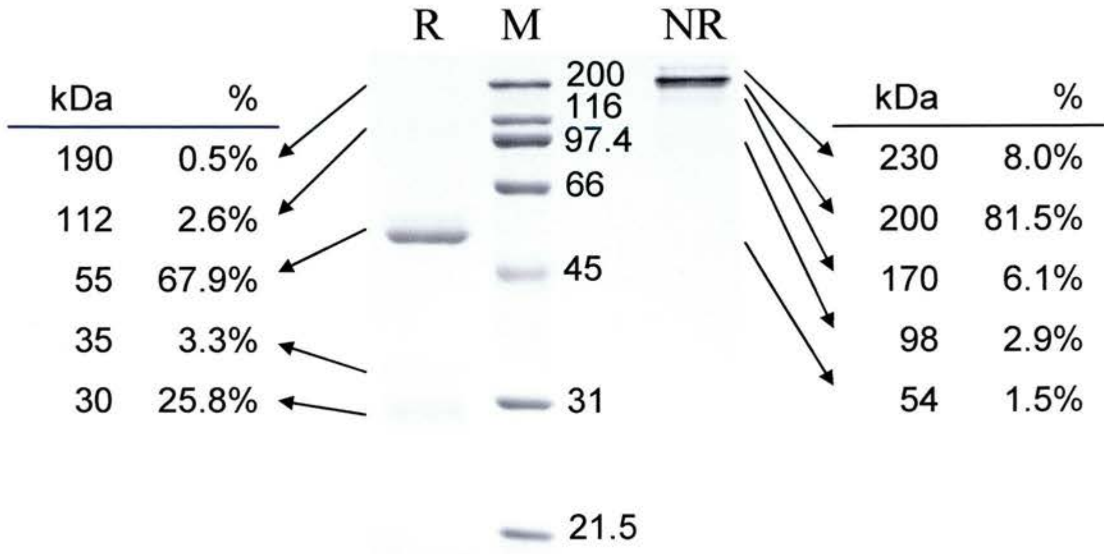
A commercial preparation of canine IgG purified by ion-exchange chromatography was used to evaluate the specificity of mAbs against canine IgG subclass antibodies.

10 μg of the purified canine IgG diluted in both reducing buffer and non-reducing buffer was separated by 10% SDS-PAGE (Chapter 2.4.2). The lane next to the sample in reducing buffer was not used because the reducing agent, 2-mercaptoethanol, could possibly diffuse to adjacent lanes and interfere with non-reducing condition. After electrophoresis, the gel was stained with Coomassie blue (Chapter 2.4.3).

Purified canine IgG separated in SDS-PAGE is shown in Figure 4.1. 55-kDa (heavy chain) and 30-kDa (light chain) proteins comprised 93.7% of the total protein content under reducing conditions, and a 200-kDa protein comprised 81.5% of the total protein under non-reducing condition. Small traces of other bands were detectable with the image analysis software, but the purity of this commercial preparation of canine IgG was considered acceptable for evaluating the specificity of mAbs.

Figure 4.1 Protein composition of the commercial preparation of canine IgG

Canine IgG was separated under both reducing and non-reducing conditions using SDS-PAGE. Molecular weight and mass percentage of individual bands are indicated. R: reducing conditions; NR: non-reducing conditions; M: molecular weight marker in kDa.



4.2.2 Assessment of the mAbs used in immunoblotting

The mAbs used in this study have previously been evaluated for their specificity against the four different IgG subclasses (Mazza and others 1993; Mazza and others 1994b). However, in order to characterise the IgG subclass responses to separated proteins from *Dermatophagoides farinae*, it was essential to demonstrate that the four different monoclonals could be used in immunoblotting experiments.

Because limited amounts of the four mAbs were available, the highest practical dilution for these antibodies was 1/200. Assay sensitivity was increased by other measures, including prolonged incubation times for antibodies and extended and repeated exposure times in recording the chemiluminescent signals.

100 μg purified canine IgG diluted in both reducing buffer and non-reducing buffer was separated by 10% SDS-PAGE using a broad well across the top (Chapter 2.4.2), transferred onto a PVDF membrane (Chapter 2.5.2) and cut into strips. After blocking non-specific binding sites with blocking buffer and thorough washing, each mAb was then added to individual strips at a dilution of 1/200 in dilution buffer and incubated overnight at room temperature. After further washing, bovine anti-mouse IgG conjugated with horseradish peroxidase at 1/1000 dilution was added to each strip before incubation at room temperature for 2 hours. The luminol substrate ECL was used to develop the chemiluminescent signals. Strips were carefully aligned and wrapped in cling film and immediately placed protein side down on the image station along with Coomassie blue stained molecular weight standards (Chapter 2.5.3).

The commercial preparation of purified canine IgG was probed with each of the four mAbs at a dilution of 1/200. Under non-reducing conditions, IgG1 gave the strongest chemiluminescent signal, followed by IgG4 (Figure 4.2 a). IgG2 and IgG3 could only be detected using less diluted mAbs (1/20) and applying a prolonged exposure of 2-hours to increase the sensitivity (compared to the 32 minutes used for IgG1) (Figure 4.2 b). Under reducing conditions, all of the four mAbs gave weak chemiluminescent signals (Figure 4.3). The reducing conditions seemed to expose epitopes that were not accessible under non-reducing conditions, and the mAbs could detect both heavy and light chains of the IgG subclass molecules.

Figure 4.2 Demonstration of binding between mAbs and purified canine IgG under non-reducing conditions

A: Purified canine IgG was probed with a panel of four mouse monoclonal antibodies specific for the subclasses of canine IgG. IgG1 gave the strongest chemiluminescent signal, followed by IgG4. B: IgG2 and IgG3 could only be detected after a prolonged exposure to increase the sensitivity. M: molecular weight standards in kDa; 1: IgG1; 2: IgG2; 3: IgG3; 4: IgG4.

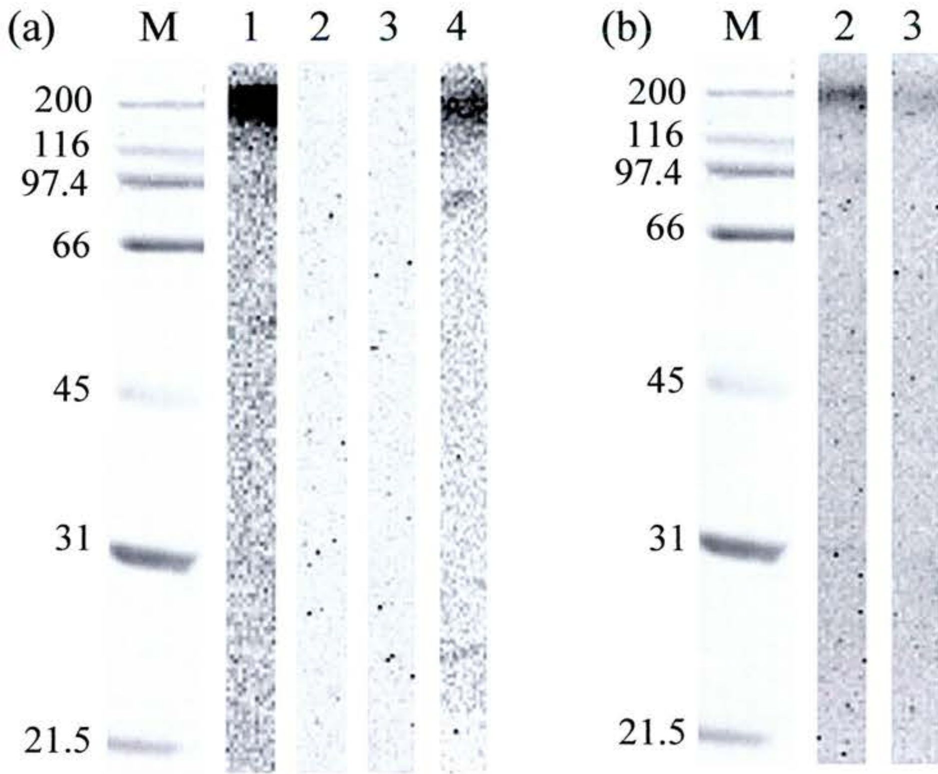
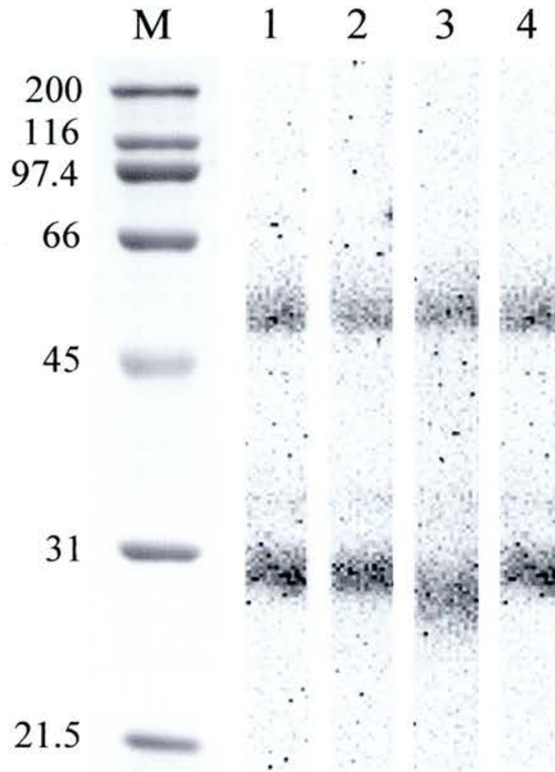


Figure 4.3 Demonstration of binding between mAbs and purified canine IgG under reducing conditions

Purified canine IgG was probed with a panel of four mouse monoclonal antibodies specific for the subclasses of canine IgG. The four mAbs were diluted in 1/200 for IgG1 and IgG4, and in 1/20 for IgG2 and IgG3. A prolonged exposure setting was applied for IgG2 and IgG3. M: molecular weight standards in kDa; 1: IgG1; 2: IgG2; 3: IgG3; 4: IgG4.



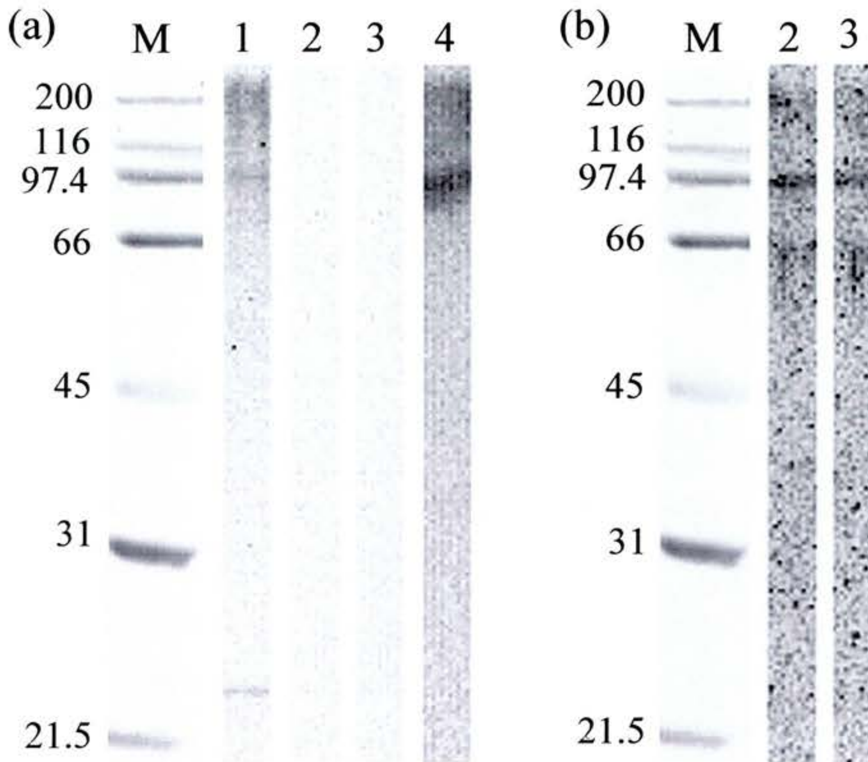
4.2.3 Demonstration of *D. farinae*-specific IgG subclass responses in immunoblots

After confirming that the four mouse monoclonal antibodies specific for the subclasses of canine IgG bound to dog IgG1, IgG2, IgG3 and IgG4, it was important to determine whether the reagents could be used to detect *D. farinae*-specific IgG subclass responses. 200 µl (1 mg/ml) crude *D. farinae* extract (Chapter 2.3) diluted in reducing buffer was separated by 10% SDS-PAGE (Chapter 2.4.2), transferred onto a membrane (Chapter 2.5.2) and cut into strips. After blocking non-specific binding sites with blocking buffer and thorough washing, a non-diluted reference serum was added and incubated for 3-4 hours. After another round of thorough washing, each mAb was then added to individual strips at a dilution of 1/100 in dilution buffer and incubated overnight at room temperature. Bovine anti-mouse IgG conjugated with horseradish peroxidase at 1/1000 dilution was added to each strip before incubation at room temperature for another 2 hours. The luminol substrate ECL was prepared and used to develop the chemiluminescent signals, and the bands were visualised using the image acquisition system described in Chapter 2.5.3.

When separated proteins from *D. farinae* were incubated with the reference canine serum sample and probed with mAbs against IgG1 and IgG4, strong signals were obtained for a protein with a molecular weight of approximately 98 kDa (Figure 4.4 a). Using the above described dilutions, incubation times and 1-2 minute exposure times, no signals were seen with IgG2 and IgG3 (Figure 4.4 a). *D. farinae*-specific IgG2 and IgG3 could only be detected when the assay sensitivity was increased dramatically by using 1/20 dilution of mAbs, and prolonged 2-hour exposure times (Figure 4.4 b).

Figure 4.4 Demonstration of *D. farinae*-specific IgG subclass responses under reducing conditions

A representative set of chemiluminescent blots showing IgG subclass responses to separated *D. farinae* antigens. Each strip was incubated with the same reference serum sample from a healthy dog and probed with mAbs against the four IgG subclasses. (a) Signals can be seen for IgG1 and IgG4 against a 98 kDa protein, but the IgG4 response is stronger. A low molecular weight band can also be seen with IgG1. No signal can be discerned with IgG2 or IgG3. (b) After prolonged incubations using low reagent dilutions, and prolonged exposures, a signal can be seen at 98 and 66 kDa with IgG2 and IgG3. M: molecular weight standards in kDa; 1: IgG1; 2: IgG2; 3: IgG3; 4: IgG4.



4.2.4 Assessment of assay reproducibility

The purpose of this section was to determine the reproducibility of the immunoblotting conditions previously determined. *D. farinae*-specific IgG2 and IgG3 were not tested here because their levels were below the detection limit using the standard protocol. 100 μg *D. farinae* extract diluted 1/1 in reducing sample buffer were separated in SDS-PAGE and transferred onto a PVDF membrane. The molecular weight marker was removed from the membrane and stained with Coomassie blue. Sixteen 4-mm wide strips were obtained and probed with the reference serum sample diluted 1/20 for 1 hour at room temperature. 8 strips were then incubated with 1/200 diluted mAb B6 (specific for canine IgG1) and 8 strips were incubated with 1/200 diluted mAb A5 (specific for canine IgG4) for 1 hour at room temperature. It was followed by another hour of incubation with 1/1000 bovine anti-mouse IgG conjugated with horseradish peroxidase and luminol solution ECL was used to visualise the bands.

Results from the eight strips detecting *D. farinae*-specific IgG1 and IgG4 respectively are shown in Figure 4.5 and Figure 4.6. The overall IgG1 and IgG4 binding profiles and the number of bands recognised were the same in all strips. Visual analysis suggested that consistent band intensity could be obtained.

The blotting results analysed by the image analysis software are summarised in Table 4.1 (IgG1) and Table 4.2 (IgG4) where data from 4 representative bands are shown. The coefficient of variation for IgG1 immunoblots ranged from 6 to 30 % for net intensity and 6 to 22 % for sum intensity. The coefficient of variation for IgG4 immunoblots ranged from 7 to 15 % for net intensity and 5 to 16 % for sum intensity.

These reproducibility figures were considered acceptable for semi-quantitative analysis.

Figure 4.5 IgG1 chemiluminescent blotting strips showing assay reproducibility

Eight *D. farinae* strips probed with the same canine reference serum sample, mAb B6, bovine anti-mouse IgG-HRP and visualised using luminol solution are shown. The overall pattern was the same in all 8 strips. Band intensity remained consistent. M: molecular weight standards in kDa.

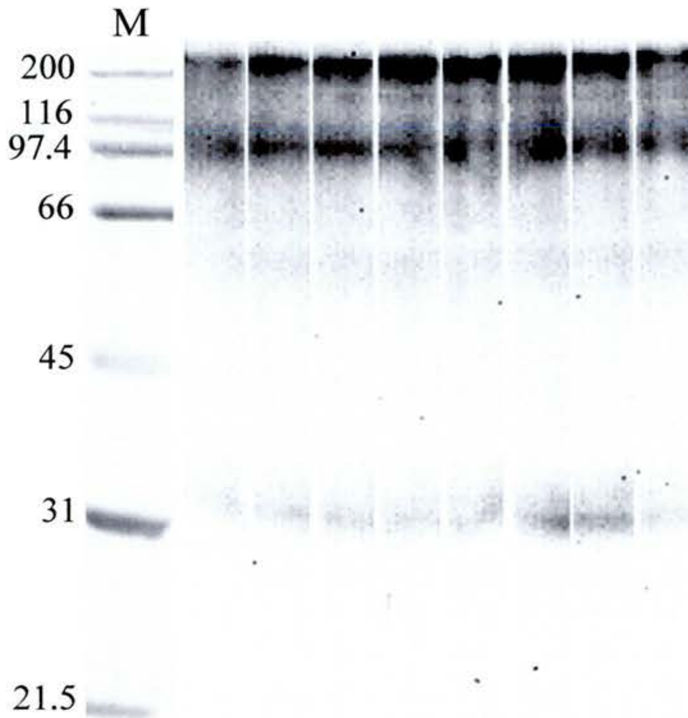


Table 4.1 Reproducibility of IgG1 immunoblotting assay

	Net intensity			Sum intensity		
Band (kDa)	μ (pixels)	σ (pixels)	Cv (%)	μ (pixels)	σ (pixels)	Cv (%)
200	92536	16157	17	103584	17935	17
97	88377	9448	11	100150	10535	11
57	22147	1310	6	32140	1941	6
33	28503	8509	30	38965	8638	22

Digital data showing net and sum intensities from 4 representative bands are summarised. The variation of band strength, measured by net intensity, did not differ from that measured by sum intensity. μ: mean; σ: standard deviation; Cv: coefficient of variation.

Figure 4.6 IgG4 chemiluminescent blotting strips showing assay reproducibility

Eight *D. farinae* strips probed with the same canine reference serum sample, mAb A5, bovine anti-mouse IgG-HRP and visualised using luminol solution are shown. The overall pattern was the same in all 8 strips. Band intensity did not differ significantly. M: molecular weight marker in kDa.

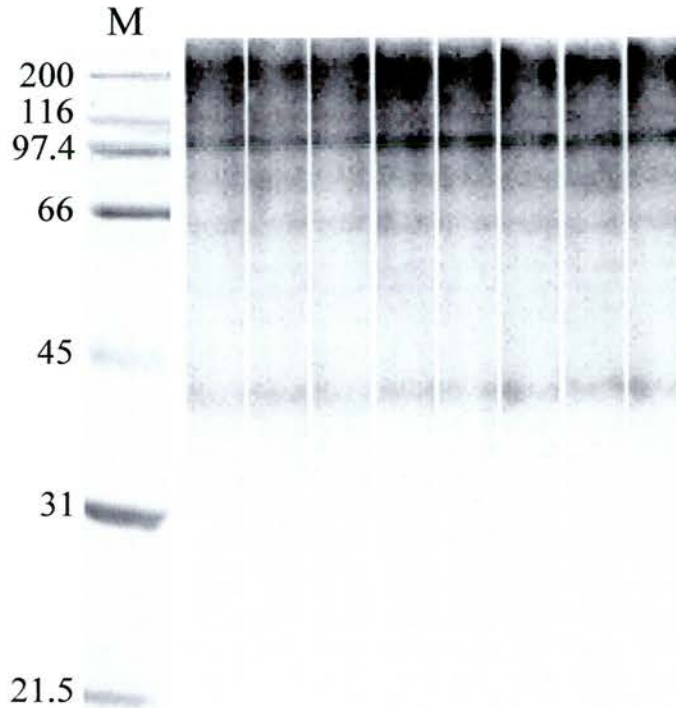


Table 4.2 Reproducibility of IgG4 immunoblotting assay

	Net intensity			Sum intensity		
Band (kDa)	μ (pixels)	σ (pixels)	Cv (%)	μ (pixels)	σ (pixels)	Cv (%)
200	148436	18924	13	161535	20765	13
97	78828	8110	10	86408	8325	10
64	30305	4657	15	36124	5684	16
44	35060	2548	7	43906	2411	5

Digital data showing net and sum intensities from 4 representative bands are summarised. The variation of band strength measured by net intensity did not differ much from that measured by sum intensity. μ: mean; σ: standard deviation; Cv: coefficient of variation.

4.2.5 Assessment of assay linearity

As with the studies on total IgG conducted in Chapter 3, it was necessary to evaluate assay linearity. The aim of this section was to determine whether changes in serum concentration resulted in a linear standard curve.

Blot strips containing separated *D. farinae* proteins were probed with 3-fold serial dilutions of the reference serum sample from 1:10 to $1:10^6$. Strips were then incubated with mAb B6 (for detection of IgG1 antibody) or mAb A5 (for detection of IgG4 antibody), followed by bovine anti-mouse IgG-HRP. *D. farinae*-specific IgG2 and IgG3 were not tested here because their levels were below the detection limit using the standard protocol. Luminol solution was used to visualise chemiluminescent signals.

Results from ten *D. farinae* immunoblotting strips are shown in Figure 4.7 (IgG1) and Figure 4.8 (IgG4). It was clear from visual observation that the more diluted the serum sample, the weaker the band signals were. *D. farinae*-specific IgG1 and IgG4 responses were not detectable in serum samples diluted more than 1000 fold.

For IgG1 immunoblots, bands with MWs of 200 kDa, 97 kDa and 34 kDa were selected and analysed by the image analysis software. For IgG4 immunoblots, bands with MWs of 200 kDa, 97 kDa and 44 kDa were selected and analysed. Results showing net intensity and sum intensity are shown in Figure 4.9 and Figure 4.10 respectively for IgG1 and IgG4 data. Linear regression could be obtained using data generated by the software. The R-squared value ranged from 0.9394 to 0.9916 for IgG1 measurements and from 0.9271 to 0.9846 for IgG4 measurements. The intensity of each band changed in a linear fashion between log serum dilution of 1/10 and 1/1000. Hence, by using serum dilutions of 1/20, the system could be used to reliably semi quantify IgG concentrations over a range that was 5× higher or 20× lower than that normally found in serum.

Figure 4.7 *D. farinae*-specific blots probed with serial dilutions of a reference serum sample followed by anti-IgG1

Separated *D. farinae* antigens transferred onto a PVDF membrane were probed with a reference canine serum sample at the following dilutions (from strip 1 to 10 in duplicate): 1×10^{-1}, 3×10^{-2}, 1×10^{-2}, 3×10^{-3}, and 1×10^{-3}. Strips probed with serum samples diluted more than 1000 fold were not shown because no signals were detected. Band signal intensity was reduced when serum concentration was decreased.

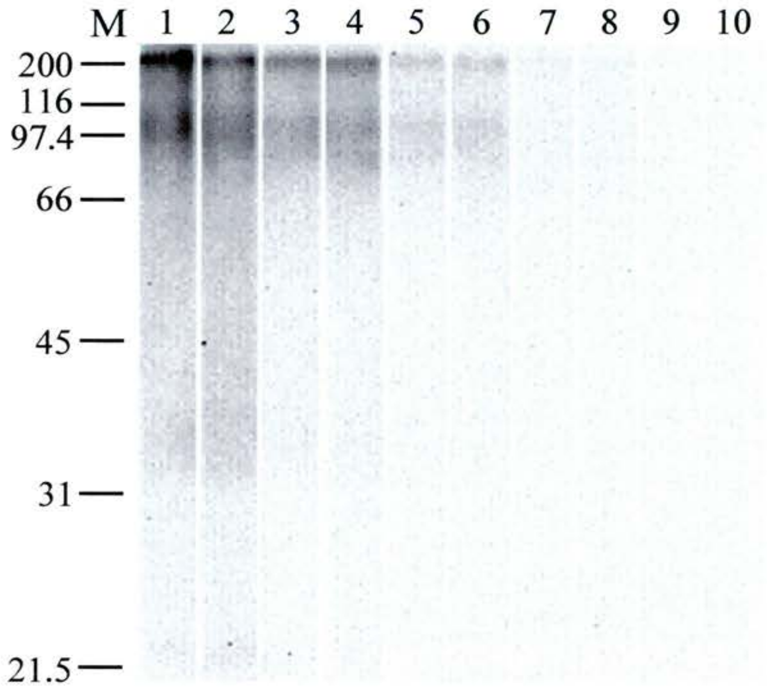


Figure 4.8 *D. farinae*-specific blots probed with serial dilutions of a reference serum sample followed by anti-IgG4

Separated *D. farinae* antigens transferred onto a PVDF membrane were probed with a reference canine serum sample at the following dilutions (from strip 1 to 10 in duplicate): 1×10^{-1}, 3×10^{-2}, 1×10^{-2}, 3×10^{-3}, and 1×10^{-3}. Strips probed with serum samples diluted more than 1000 fold were not shown because no signals were detected. Band signal intensity was reduced when serum concentration was decreased.

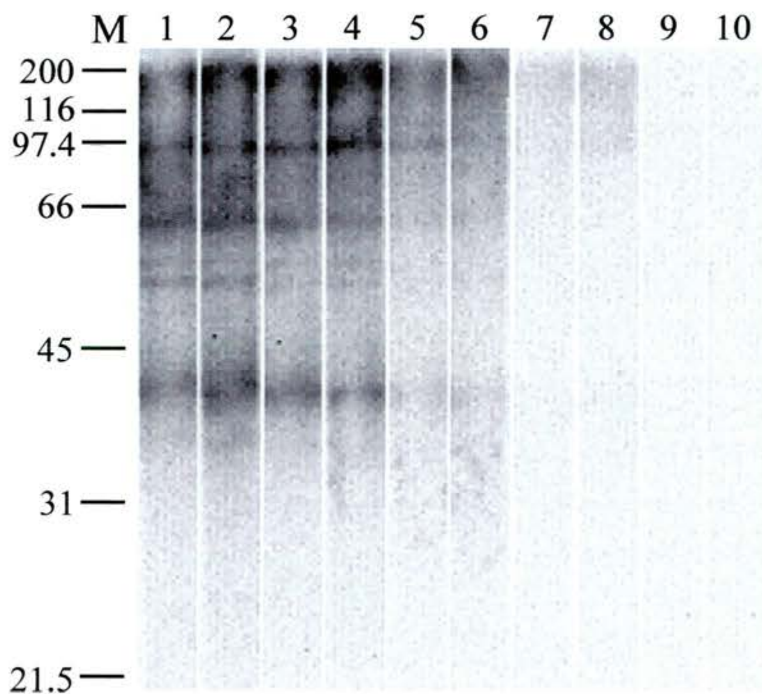


Figure 4.9 Demonstration of linear relationship between signal intensity and serum concentration measuring *D. farinae*-specific IgG1

The X axis represents serum concentration (μg/ml) in log scale while the Y axis represents intensity ($\times 10^4$ pixels). Graphs on the left indicate net intensity and graphs on the right indicate sum intensity. Logarithmic trend lines and R-squared values are shown on each graph. Linear regression fit was observed.

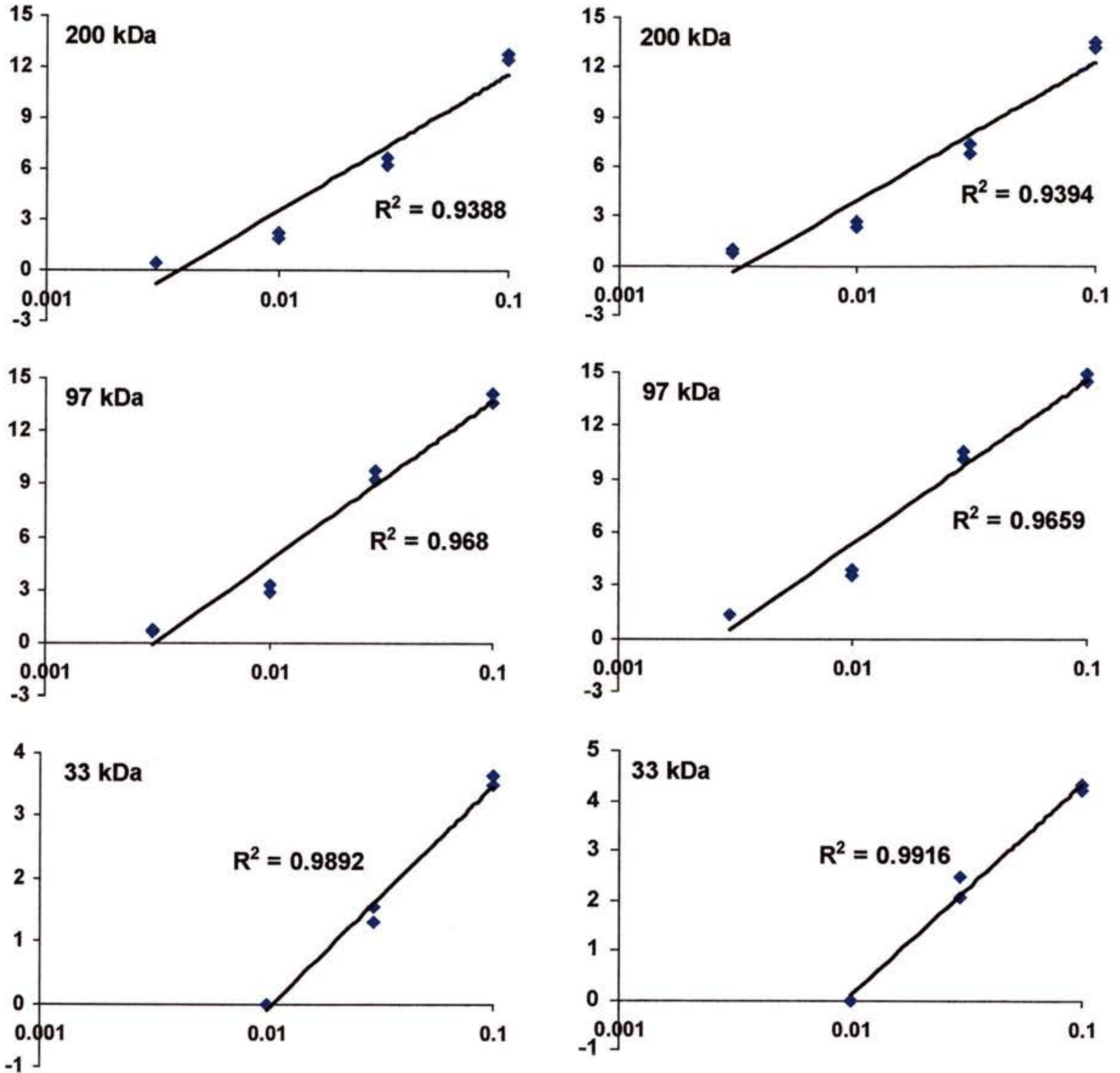
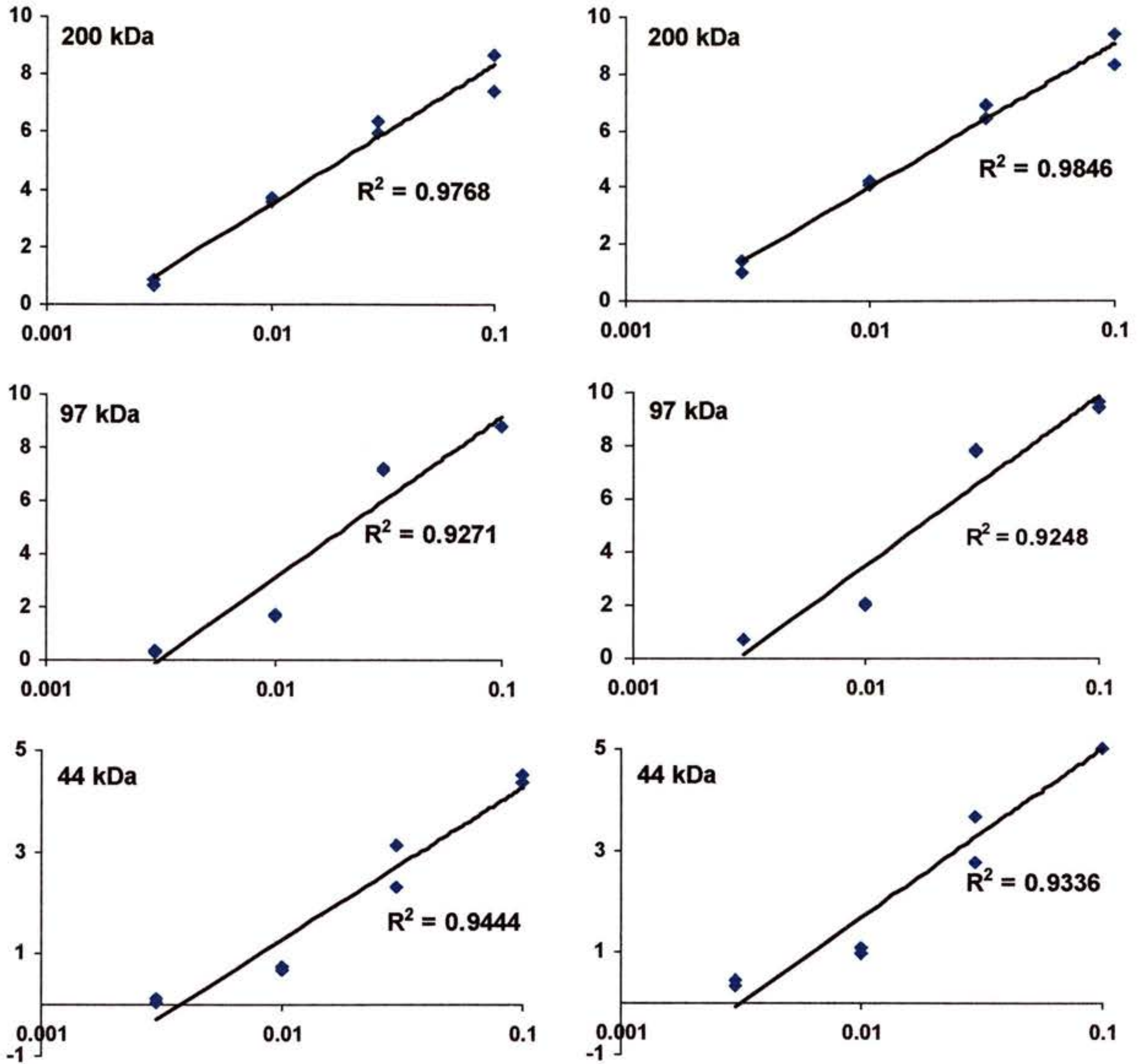


Figure 4.10 Demonstration of linear relationship between signal intensity and serum concentration measuring *D. farinae*-specific IgG4

The X axis represents serum concentration (μg/ml) in log scale while the Y axis represents intensity ($\times 10^4$ pixels). Graphs on the left indicate net intensity and graphs on the right indicate sum intensity. Logarithmic trend lines and R-squared values are shown on each graph. Linear regression fit was observed.



4.3 Dermatophagoides farinae-specific IgG subclass responses in healthy and atopic dogs

The objective of this section was to use the previously validated semi-quantitative blot analysis system to characterise and quantify the *D. farinae*-specific IgG subclass responses both in healthy and atopic dogs.

Serum samples collected from 20 healthy dogs (Chapter 2.1.2) and 20 atopic dogs (Chapter 2.1.3) were included in this study. 100 μg (1 mg/ml) *D. farinae* extract diluted 1/1 in reducing sample buffer was separated in 10% SDS-PAGE gel and electrophoretically transferred onto a PVDF membrane. The molecular weight standards were cut off and stained with Coomassie blue. The rest of the membrane was cut into 16 4-mm wide strips to facilitate subsequent probing with different serum samples and non-specific binding sites were blocked with 5% skimmed milk in TBS for 1 hour. To minimise inter-membrane differences, 8 strips were used to detect *D. farinae*-specific IgG responses from healthy dogs and another 8 strips from the same membrane were used to detect that from atopic dogs. For immunoblotting, 1 ml of 1/20 diluted serum samples was added to individual strips for 1 hour at room temperature. After thorough washing, strips were incubated with 1 ml of 1/200 diluted mAb B6 for IgG1 detection or 1ml of 1/200 diluted mAb A5 for IgG4 detection for another hour at room temperature. After another round of thorough washing with frequent changes of washing buffer, individual strips were incubated with 1 ml of 1/1000 diluted bovine anti-mouse IgG conjugated with horseradish peroxidase for 1 hour. Bands were visualised using luminol substrate ECL and recorded on the image station. The optimal exposure settings were 32 × 1 min exposures for IgG1 blots and 32 × 20 sec exposures for IgG4 blots. Exposure time was estimated based on the acquired preview image of the blots and repeated exposure was applied to eliminate potential saturation phenomena.

Results were studied using the Image Analysis software (Chapter 2.5.3), and data were then exported to MINITAB™ statistical software (Minitab Inc., PA, USA) for analysis. The Mann-Whitney test was used to compare healthy and atopic dogs for the number of bands recognised, the molecular weight range of the bands observed,

the total intensity of the bands in each group and the intensity for the most important individual bands. A p-value of <0.05 was considered to be significant.

Both healthy and atopic dogs mounted detectable *D. farinae*-specific IgG1 and IgG4 responses (Figure 4.11 and Figure 4.12). Both IgG1 and IgG4 recognised multiple proteins from *D. farinae* with the most visually obvious bands having molecular weights of approximately 98 kDa and 18 kDa for IgG1 (Figure 4.11) and 98 kDa and 45 kDa for IgG4 (Figure 4.12).

The IgG1 chemiluminescent blots of all dog sera from the two groups are shown in Figure 4.11. The intensity of each band, which represents the magnitude of the IgG response to that particular protein, is summarised in Figure 4.13. The most commonly recognised allergen was the 98 kDa protein, followed by a low molecular weight antigen of 18 kDa. The number of bands recognised per dog did not differ significantly between the two groups ($p=0.99$) and varied between 1 and 8 in the healthy group and 2 and 6 in the atopic group, with median band numbers of 3.5 and 3 respectively. The sum of all the band intensities in each group (i.e. the total IgG1 response to all *D. farinae* allergens) was 85616 in the healthy group and 65094 in the atopic group ($p=0.6879$). The responses to the 98 kDa and 18 kDa antigens were slightly higher in the healthy group (intensities 21947 and 20197) than in the atopic group (intensities 19368 and 16344) but the difference did not reach statistic significance ($p=0.5188$ for 98 kDa protein and $p=0.1148$ for 18 kDa antigen) (Figure 4.15).

The IgG4 chemiluminescent blots of all dog sera from the two groups are shown in Figure 4.12, and the data summarised graphically in Figure 4.14. As with IgG1, a 98 kDa protein was commonly detected in both groups, whereas an antigen with a molecular weight of approximately 45 kDa was also present. The number of bands recognised did not differ between the two groups ($p=0.5975$) and varied between 1 to 9 in the healthy group and 1 to 11 in the atopic group, with median band numbers of 6 and 4 respectively. The sum of all the band intensities in each group (i.e. the total IgG4 response to all *D. farinae* allergens) was 106700 in the healthy group and

87813 in the atopic group (p=0.3123). The responses to the 98 kDa and 44 kDa antigens were slightly higher in the healthy group (intensities 21242 and 16967) than in the atopic group (intensities 18758 and 15690) but these were not significantly different (p=0.1117 for 98 kDa protein and p=0.6893 for 45 kDa antigen) (Figure 4.16).

Figure 4.11 Binding pattern of canine IgG1 to separated proteins of *D. farinae* in 20 healthy dogs (a) and 20 atopic dogs (b)

Each strip represents one dog. Similar IgG binding profiles were observed. The most visually obvious bands were 98 kDa and 18 kDa antigens. M: molecular weight standards in kDa.

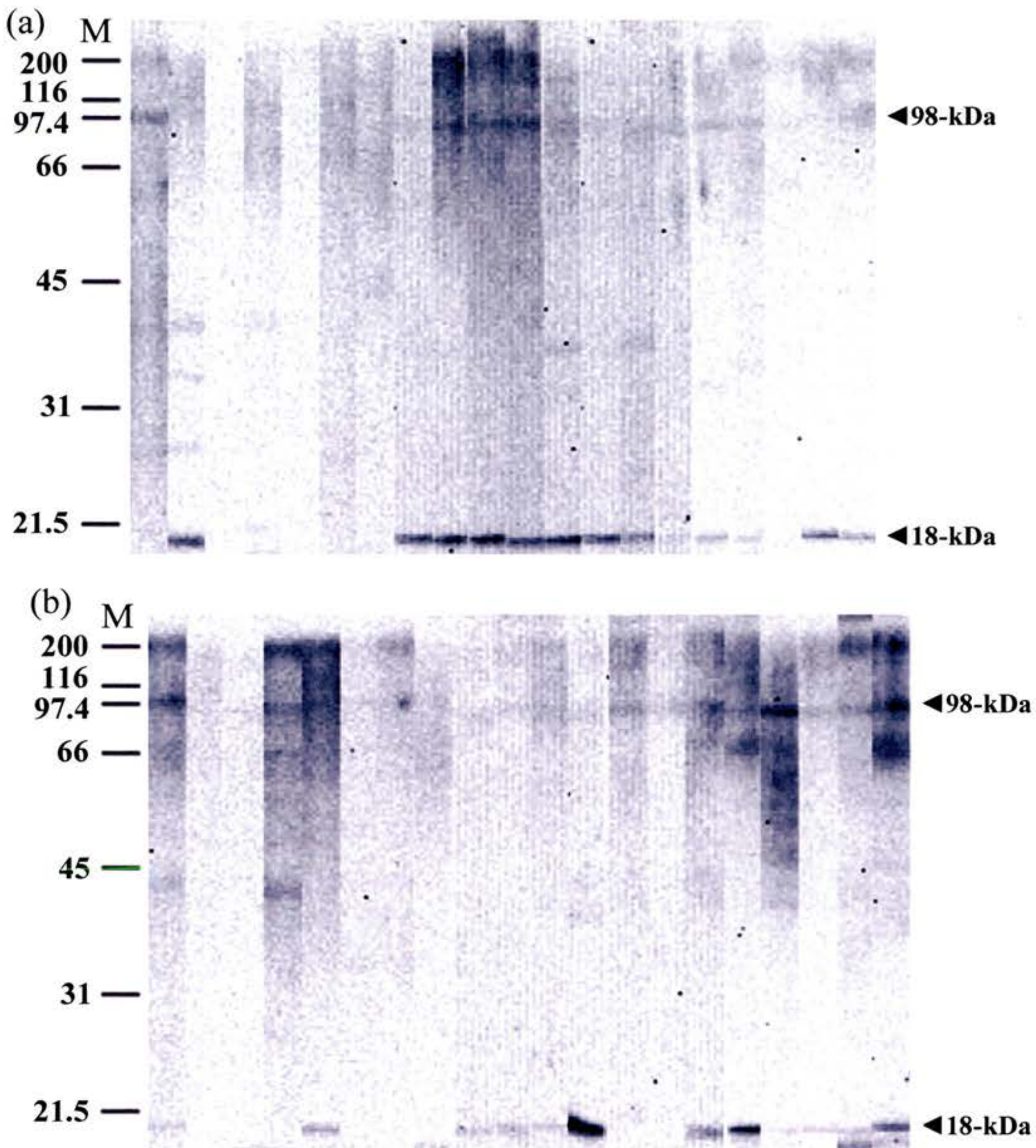


Figure 4.12 Binding pattern of canine IgG4 to separated proteins of *D. farinae* in 20 healthy dogs (a) and 20 atopic dogs (b)

Each strip represents one dog. Similar IgG binding profiles were observed. The most visually obvious bands were 98 kDa and 44 kDa antigens. M: molecular weight standards in kDa.

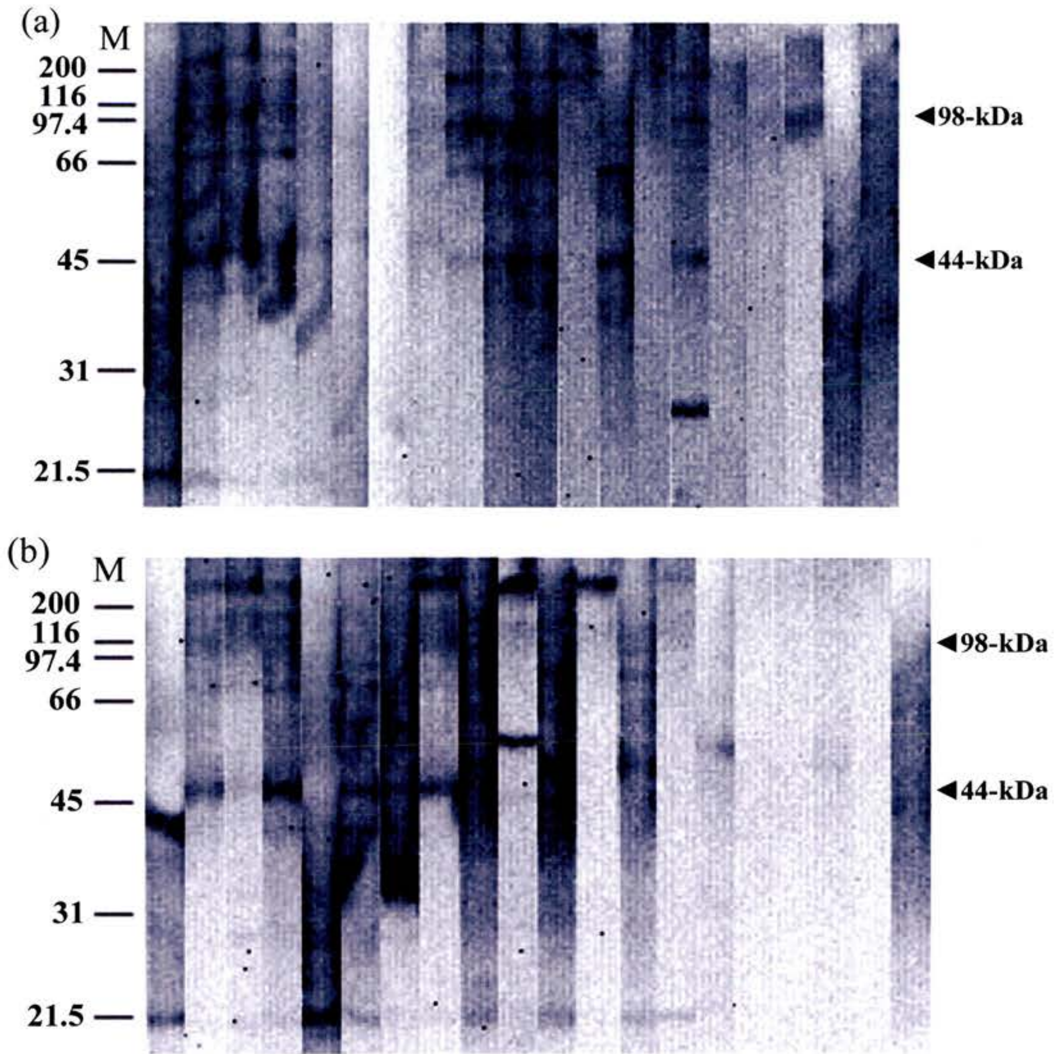


Figure 4.13 Scatter plot of band molecular weight against *D. farinae*-specific IgG1 band intensity in healthy and atopic dogs

Each dot represents an IgG1 response to a *D. farinae* protein in an individual dog.

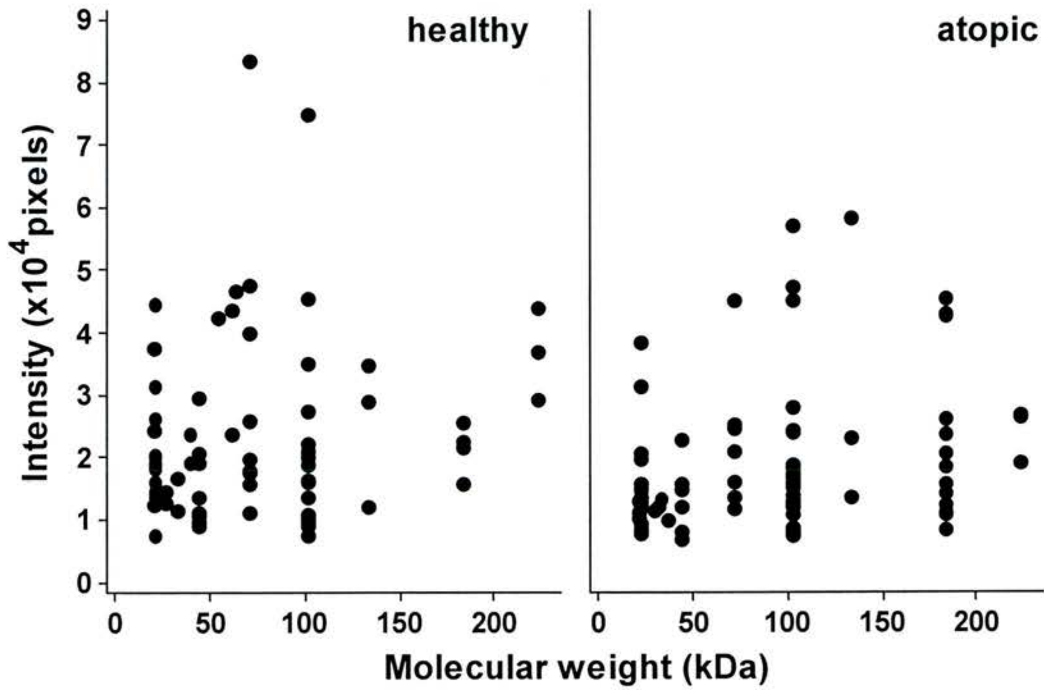


Figure 4.14 Scatter plot of band molecular weight against *D. farinae*-specific IgG4 band intensity in healthy and atopic dogs

Each dot represents an IgG4 response to a *D. farinae* protein in an individual dog.

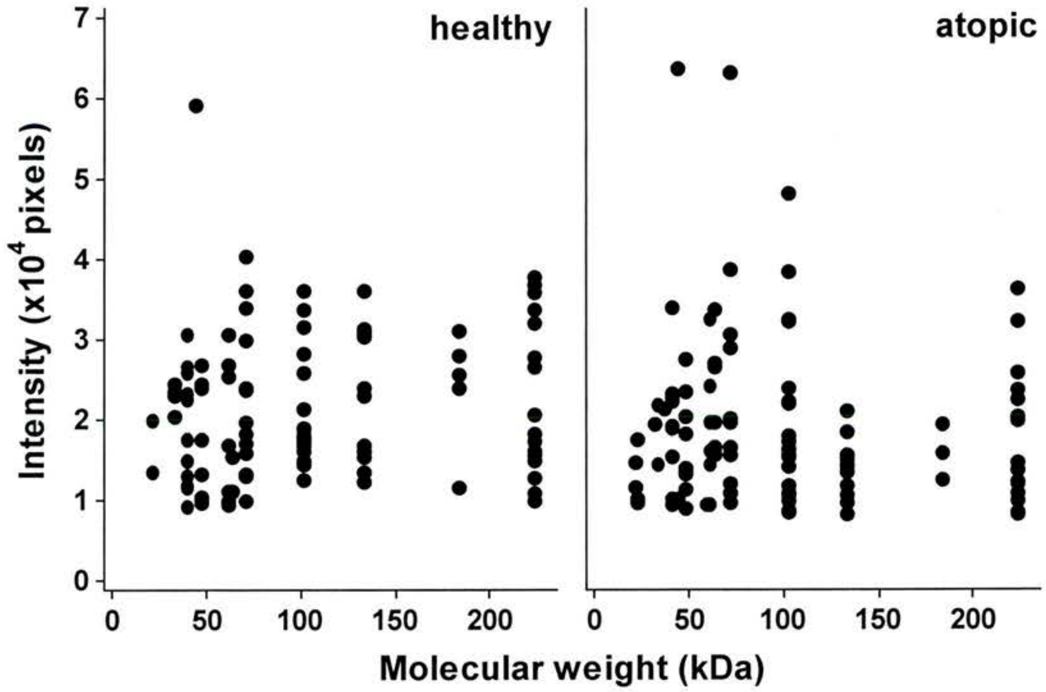


Figure 4.15 Intensity of the IgG1 response to the two most commonly recognised antigens in *D. farinae*

The box and whisker plots summarise the response of 20 atopic and 20 normal dogs to proteins with molecular weights of 98 kDa and 18 kDa. The middle bar represents the median, the upper and lower bars represent the 25th and 75th percentiles, and the whiskers extend to the highest or lowest values that fall within 1.5 times the interquartile range. * represents outlier.

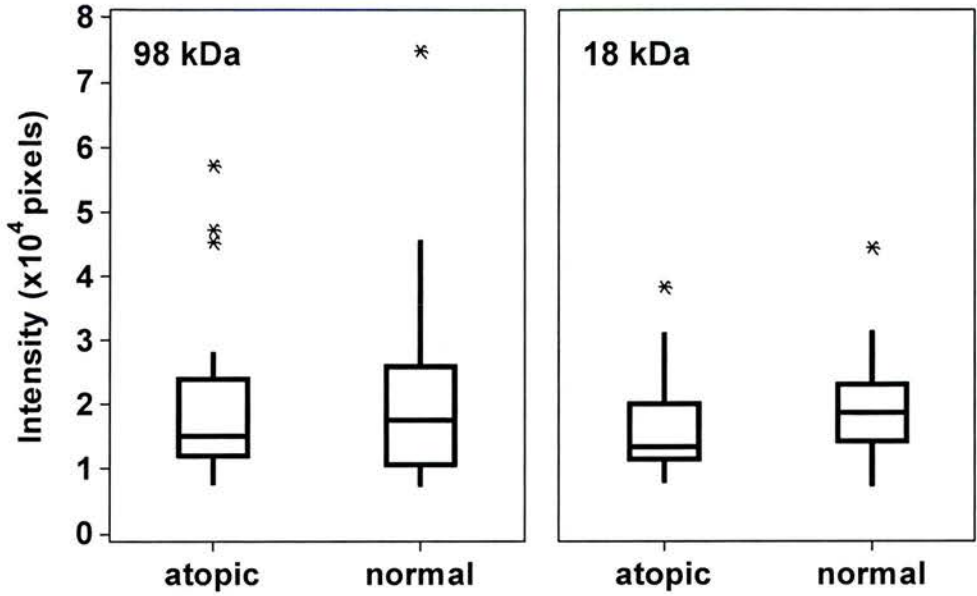
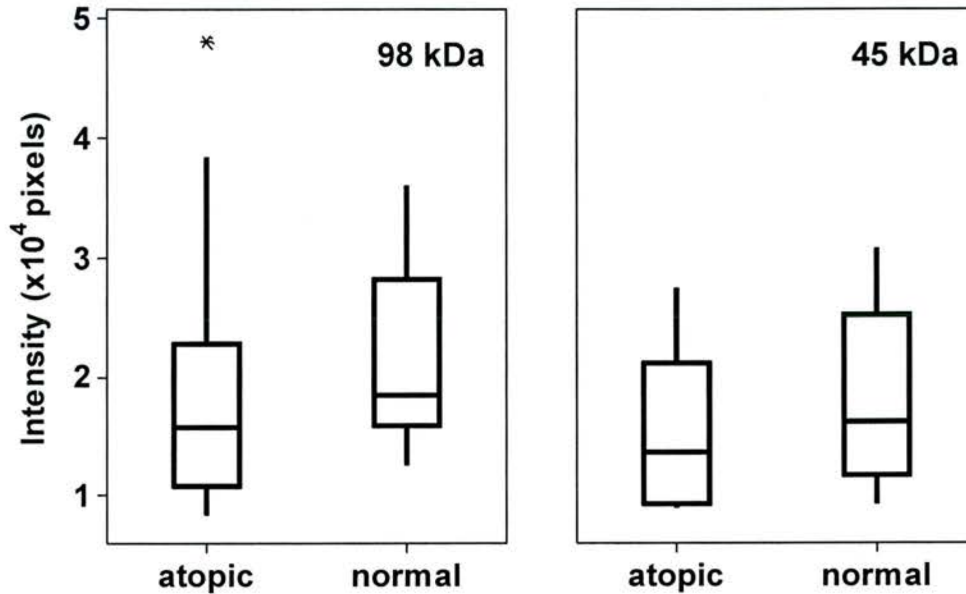


Figure 4.16 Intensity of the IgG4 response to the two most commonly recognised antigens in *D. farinae*

The box and whisker plots summarise the response of 20 atopic and 20 normal dogs to proteins with molecular weights of 98 kDa and 45 kDa. The middle bar represents the median, the upper and lower bars represent the 25th and 75th percentiles and the whiskers extend to the highest or lowest values that fall within 1.5 times the interquartile range.* represents outlier.



4.4 Discussion

In this chapter we aimed to further develop and validate the semi-quantitative western blot analysis system to allow us to investigate the IgG subclass responses to *D. farinae* allergens in both healthy and atopic dogs. Initially, we determined if the four mAbs specific for the subclasses could recognise the appropriate antibodies in immunoblots using a commercially available whole IgG preparation. The signals for IgG1 and IgG4 were much stronger than for IgG2 and IgG3 (Figure 4.2). This does not reflect the serum levels of the four subtypes as reported by Mazza (Mazza and others 1994a), where IgG1 and IgG2 were shown to have at least nine fold higher concentrations than IgG3 and IgG4. One explanation for this potential discrepancy is that the whole IgG used in these validation experiments may have had concentrations of subclasses within it that do not mirror serum concentrations. Alternatively, the results may indicate that the monoclonal antibodies used for IgG2 and IgG3 were less able to bind to immunoblots compared to the ELISA assay used by Mazza (Mazza and others 1994a) leading to lower signal strengths. This would lower the sensitivity of the immunoblotting system to detect IgG2 and IgG3 responses.

When the purified canine IgG was denatured by heating in the presence of the reducing agent β-mercaptoethanol, the non-covalent intra- and inter-molecular associations, i.e. disulfide bridges, were disrupted and the IgG was effectively rendered devoid of secondary and tertiary structure. Under denaturing conditions, two bands corresponding to immunoglobulin heavy chain and light chain were detected. However, dog IgG2 and IgG3 could only be detected when the assay sensitivity was dramatically increased by using less diluted mAbs and prolonged exposure settings (Figure 4.3)

When the immunoblotting system was used to detect IgG subclass responses to separated *D. farinae* proteins, strong signals could only be obtained for IgG1 and IgG4. IgG2 and IgG3 responses were virtually undetectable, unless very long incubation and exposure times were used, together with low dilutions of sera and reagents. The lack of an IgG2 and IgG3 response could be due to poor binding of these reagents in this type of system as suggested above. It could also reflect the

possibility that IgG2 and IgG3 responses are minimal against environmental antigens such as those from *D. farinae*, as suggested by Day (Day and others 1996).

Densitometric analysis of chemiluminescent protein signals revealed that the reproducibility of *D. farinae*-specific IgG1 and IgG4 responses as assessed by coefficient of variation ranged from 5 to 30%. Chemiluminescent application was chosen because of its superior lower detection limit, but reliable measurements were difficult to obtain because photon emission intensity declined with time, repeated long exposure was applied in order to capture faint chemiluminescent signals whilst avoiding potential saturation of strong bands, and minute signal intensity variation due technical limitation of cooled CCD camera recording system. A coefficient of variation of below 10% of the detection system was generally considered of high reproducibility. Despite the substandard repeatability of our chemiluminescence assay, the coefficient of variation figures was considered acceptable for semi-quantitative analysis.

The IgG1 and IgG4 responses to *D. farinae* proteins allowed linear standard curves to be generated. This allowed semi-quantitative analysis of the antibody concentrations in serum at concentrations approximately 20 times lower than the concentrations measured in the reference sample, similar to the results found when measuring total IgG responses (Chapter 3).

Using the Western blot analysis system, we compared allergen-specific IgG1 and IgG4 subclass responses to various antigens of the house dust mite *D. farinae* in healthy dogs and dogs with atopic dermatitis. The profiles of the IgG1 and IgG4 responses were similar in the two groups, both in terms of the number of bands recognised, and their molecular weight. The percentage of individual dogs recognising these proteins was also similar in the two groups. These results indicate that *D. farinae* produces a number of proteins that are recognised by the canine immune system, both in healthy and atopic dogs, and are in accordance with previous findings (Lian and Halliwell 1998; Hou and others 2005). However, studies by Day et al (Day and others 1996) only detected antigen-specific IgG antibodies against *D.*

farinae using ELISA in one out of eleven normal dogs. The reason for this discrepancy is not known but it might arise from technical, geographical, environmental or gene pool differences in healthy dogs included in the respective studies.

In terms of the proteins recognised, there was both an IgG1 and IgG4 response to an antigen with a molecular weight of approximately 98 kDa. This is likely to be the high molecular weight allergen Der f 15 that is recognised by IgE in atopic dogs (Noli and others 1996; McCall and others 2001; Nuttall and others 2001b). IgG1 also recognised a low molecular weight protein of approximately 18 kDa, whereas IgG4 commonly recognised an antigen with a molecular weight of approximately 45 kDa. A similar protein is also recognised by IgE in immunoblots (Nuttall and others 2001b). These findings suggest that particular allergens can stimulate different subclass responses.

The regulatory mechanisms of IgG subclass production to specific allergens has not yet been fully determined. It has been shown that IgG1 and IgG4 antibodies to grass pollen in allergic patients are synchronised with seasonal variation of IgE antibody in response to seasonal pollen exposure (Nordvall and others 1986). Studies investigating IgG subclasses in association with dust mite allergen levels has suggested that seasonal changes in natural exposure to house dust mite allergen might lead to concurrent changes in *D. farinae*-specific IgG4 in mite-sensitive patients (Nahm and others 1998). In terms of antigen-specific IgG subclass responses, it has been shown that *D. pteronyssinus* group II (Der p 2) antigen was able to elicit increased Der p 2-specific IgG4 responses in patients with mite allergy (Tame and others 1996). Another study has demonstrated that levels of *D. farinae* group II (Der f 2)-specific IgG2 and IgG4 were higher in the atopic group, but the reverse was found for the *D. farinae* group I (Der f 1)-specific IgG2, meaning significantly higher concentration of Der f 1-specific IgG2 was found in the non-atopic group (Hong and others 1994). Taken together, the results suggest that specific proteins in *D. farinae* are able to stimulate specific subclass responses, but further studies are required to determine how this happens.

The strength of the IgG1 and IgG4 responses was generally slightly higher in the healthy controls than in the atopic dogs, both overall and for the most intense bands. The lack of significant differences between the groups might imply that this difference was biologically irrelevant. However, a study including a larger number of dogs in each group might help to clarify whether the difference was genuine and relevant. Hill et al (Hill and others 1995) reported that total IgG concentrations were significantly higher in atopic and parasitized dogs compared to healthy controls. However, this study did not measure allergen-specific IgG, or IgG subclasses, and the antibody response could have been directed against a range of antigens, including those derived from staphylococci and *Malassezia* organisms (Nuttall and Halliwell 2001a; Chen and others 2002a).

Although it is not possible to accurately compare the strength of the IgG1 response to the IgG4 response because we do not know the exact concentration or affinities of the mAbs, there is some evidence to suggest that the IgG4 response to *D. farinae* antigens was stronger. The IgG4 concentration in normal dog serum is 9 times less than the IgG1 response and yet in these experiment, the signals on the blots were much stronger (Compare Figures 4.11 and 4.12), even when using shorter exposure times.

Taken together, the results of this and previous studies suggest that *D. farinae*-specific IgG and IgG subclasses do not play a major role in the pathogenesis of canine atopic dermatitis. Such responses occur in both healthy and atopic dogs and do not appear to be either protective or pathogenic.

Chapter 5

Dermatophagoides farinae-specific immunoglobulin G responses in atopic dogs undergoing allergen-specific immunotherapy using alum-precipitated or aqueous vaccines

5.1 Introduction

Allergen-specific immunotherapy (ASIT) is the practice of administering gradually increasing quantities of an allergen extract to an allergic subject to ameliorate the symptoms associated with subsequent exposure to the causative allergen (Bousquet and others 1998; Griffin and Hillier 2001b). The efficacy of ASIT has been well documented for humans with respiratory atopic diseases and stinging insect allergy, but its effectiveness seems more controversial for patients with AD. Since Wittich (Wittich 1941) first reported in 1941 that ASIT could be used successfully in the treatment of seasonal pollinosis in dogs, many studies, and a large body of clinical observations by veterinary dermatologists, have suggested that ASIT can be effective in controlling the signs of AD in dogs (Nesbitt 1978; Willemse and others 1984; Scott and Miller 1993c; Mueller and Bettenay 1996; Nuttall and others 1998; Zur and others 2002b). However, most of these data are either anecdotal or originate from questionnaire-based studies, and, to date, there has been only one placebo-controlled study that has investigated this form of treatment in dogs (Willemse and others 1984). Collectively, this body of information and literature has resulted in efficacy claims ranging from 50% to 100%, with a response usually being defined as in improvement in clinical signs of at least 50% (Griffin and Hillier 2001b).

Two main forms of ASIT vaccines are currently used in dogs: aqueous vaccines are favoured in North America whereas alum-precipitated vaccines are commonly used and recommended in Europe (Willemse and others 1984). A comparison of the true efficacy of these vaccine types is not possible because different studies quote differences in allergen extracts, the number of allergens used, the concentration of allergens, the frequency of administration, as well as assessment of clinical outcome

which is highly subjective and easily influenced by other factors such as secondary infections. One potential advantage of aluminium-adjuvanted vaccines is the more rapid development of high titre and long-lasting antibody responses after primary immunisation (Volk and Bunney 1942). The mechanism of action of aluminium adjuvant likely involves the formation of a depot, increasing targeting of antigens to antigen-presenting cells, and non-specific activation of the immune response (Gupta 1998). However, in human patients with Hymenoptera venom allergy, the therapeutic efficacy of specific immunotherapy using aluminium hydroxide adsorbed vaccines and aqueous preparations did not differ (Rueff and others 2004).

Although the clinical efficacy of ASIT is well documented in humans and dogs, the molecular and immunological mechanisms involved are incompletely understood. Current evidence from rodent and human studies suggests that ASIT exerts effects on several aspects of the immune system, including modulation of allergen-specific B cells as well as T-cell responses. Studies on the effect of ASIT have demonstrated reduced basophil reactivity to allergens (Kimura and others 1985; Shim and others 2003), deviation of Th2-cytokine responses to allergens in favour of the Th1 responses (Secrist and others 1993; Jutel and others 1995; McHugh and others 1995; Akoum and others 1996), and the induction of IL-10-producing regulatory T cells (Bellinghausen and others 1997; Akdis and others 1998a; Francis and others 2003). In addition, changes in serum antibody titres in response to ASIT have been described, mostly as increases in allergen-specific IgG antibodies, particularly of the IgG1 and IgG4 isotypes (Muller and others 1989; McHugh and others 1990). Allergen-specific IgG produced in response to ASIT has been proposed as "blocking antibody" by competing with IgE for allergen binding to mast cells, basophils, and other IgE receptor-expression cells.

To date, only two studies investigating changes in IgG concentrations have been reported in dogs during ASIT (Hites and others 1989; Fraser and others 2004). Hites et al reported increases in allergen-specific IgG antibodies to various pollens during ASIT using aqueous allergens (Hites and others 1989). Fraser et al showed that concentrations of total IgG1 could increase following ASIT using adjuvanted

vaccines (Fraser and others 2004), but this study did not investigate allergen-specific responses. However, no studies have reported changes in IgG antibodies specific for antigens derived from the house dust mite *Dermatophagoides farinae*, a major cause of AD in dogs (Noli and others 1996; Hill and DeBoer 2001; McCall and others 2001; Nuttall and others 2001b).

The aim of these studies was to use the semi-quantitative blot analysis system to investigate changes in IgG to separated antigens from *D. farinae* during ASIT with alum-precipitated and aqueous vaccines. Our hypothesis was that allergen-specific IgG antibodies would increase after the administration of allergen-specific immunotherapy in dogs with atopic dermatitis.

5.2 Study 1 – ASIT using alum-precipitated vaccines

5.2.1 Material and methods

5.2.1.1 Serum samples

Serum samples were obtained from 21 dogs with AD taking part in a clinical trial to evaluate the efficacy of two different ASIT protocols using adjuvanted vaccines (Colombo and others 2005). All dogs included had a positive reaction to *D. farinae* tested by either intradermal test (IDT) or allergen-specific IgE serology (ASIgES) (Chapter 2.1.4). Details of the inclusion and exclusion criteria, study design, clinical assessment and scoring, use of concurrent medications and analysis of treatment efficacy, are reported elsewhere (Colombo and others 2005). Serum samples were to be collected from each dog before and after 3, 6 and 9 months of ASIT. However, due to unavoidable logistical and technical circumstances, serum samples could not be collected at every time point in every dog. All dogs were sampled prior to the start of ASIT but some only had serum samples taken at one or two of the remaining three time points. There were 4/24 missing data points in the complete response group, 5/20 in the partial response group and 5/40 in the no response group.

All samples were obtained with the owner's consent. As the samples were to be used for the measurement of allergen-specific antibody responses, which represented diagnostically and therapeutically useful information; UK Home Office approval was not required. After collection, serum was separated by centrifugation and stored in aliquots at -20°C until used (Chapter 2.2.1).

5.2.1.2 Immunotherapy protocol

An alum-precipitated vaccine (Artuvetrin® Therapy, ARTU, The Netherlands) containing a mixture of allergens specific for each dog and formulated based on the results of IDT or ASIgES (Chapter 2.1.4), was used in all cases. The dogs were allocated to two treatment groups according to block randomisation. The control groups (standard dose, SD) consisted of ten dogs receiving ASIT following the standard protocol as indicated by the manufacturer (Table 5.1). This involved administration of an allergen mixture comprising up to eight allergens, at least one of which was *D. farinae* which accounted for 2.5-10% v/v of the allergen mixture. The experimental group (low dose, LD) consisted of eleven dogs treated with ASIT but receiving 0.1 ml only of the vaccine at the same standard intervals (Table 5.1). The precise concentration of allergens is not provided by the manufacturer.

Table 5.1 Protocols of allergen-specific immunotherapy using alum-precipitated vaccine in the low dose and standard dose groups

Injection	Week	Dose of allergen extract (ml)		Site
		Standard-dose ASIT	Low-dose ASIT	
1	0	0.2 ml	0.1 ml	L Neck
2	2	0.4 ml	0.1 ml	R Neck
3	4	0.6 ml	0.1 ml	R Flank
4	6	0.8 ml	0.1 ml	L Flank
5	9	1.0 ml	0.1 ml	L Neck
6*	12	1.0 ml	0.1 ml	R Neck
7	16	1.0 ml	0.1 ml	R Flank
8	20	1.0 ml	0.1 ml	L Flank
9*	24	1.0 ml	0.1 ml	L Neck
10	28	1.0 ml	0.1 ml	R Neck
11	32	1.0 ml	0.1 ml	R Flank
12*	36	1.0 ml	0.1 ml	L Flank

SD: standard dose; LD: low dose; *: reinspection at the R(D)SVS and blood sample collected if possible.

5.2.1.3 Assessment of pruritus

Dogs were assessed for pruritus by the owners using a 0-5 point behaviour-based scale (Table 5.2) and for skin lesions by a single investigator (S. Colombo) using a modified canine atopic dermatitis extent and severity index (CADESI) (Table 5.3) (Colombo and others 2005). Assessments took place on the day the ASIT was started and after 3, 6 and 9 months (90, 180 and 270 days) of ASIT. A complete response to ASIT was defined as a dog whose pruritus score had fallen to zero by the end of the study without the need for additional anti-pruritic medication. A partial response to ASIT was defined as a dog whose pruritus score was lower at the end of the 9-month treatment period compared to the beginning. No response to ASIT was defined as a dog whose pruritus score was the same or higher after 9 months of treatment compared to the beginning. Glucocorticoid therapy was permitted for the first 6 months of the study, but not during the final 3 months of ASIT.

Table 5.2 0-5 point behaviour-based pruritus scale

Grade	Description
0	**Normal dog**: the dog does not itch more than before the disease began.
1	**Occasional episodes** of itching (small increase in itch compared with before the disease began).
2	**More frequent episodes** of itching, but the itching stops when the dog is sleeping or eating or playing or exercising or is otherwise distracted.
3	**Regular episodes** of itching are seen when the dog is awake. The dog occasionally wakes up because of itching, but the itching stops when the dog is eating or playing or exercising or is otherwise distracted.
4	**Prolonged episodes** of itching are seen when the dog is awake. The dog regularly wakes up because of itching, or itches in its sleep. The itching can also be seen when the dog is eating or playing or exercising or is otherwise distracted.
5	**Almost continuous itching** which does not stop when the dog is distracted, even in the consulting room (the dog needs to be physically restrained from itching).

N.B.: Itching includes scratching, biting, licking, chewing and rubbing.

Table 5.3 Modified canine atopic dermatitis extent and severity index (CADESI)

Sites	Erythema	Hyperpigmentation	Lichenification	Papular/Pustular Eruption	Greasy seborrhea
Muzzle					
Head					
Left ear					
Right ear					
Dorsal neck					
Ventral neck					
Dorsum					
Left axilla					
Right axilla					
Left flank					
Right flank					
Abdomen					
Left groin					
Right groin					
Perineum					
Tailhead					
Left fore:					
Upper limb					
Distal limb					
Metacarpal					
Dorsal foot					
Ventral foot					
Right fore:					
Upper limb					
Distal limb					
Metacarpal					
Dorsal foot					
Ventral foot					
Left hind:					
Upper limb					
Distal limb					
Metacarpal					
Dorsal foot					
Ventral foot					
Right hind:					
Upper limb					
Distal limb					
Metacarpal					
Dorsal foot					
Ventral foot					
Total score:					

Pruritus score: 0 1 2 3 4 5

Pyoderma: Yes No

Sites checked: ………………   Sites affected: ………………

Malassezia dermatitis: Yes No

Sites checked: …………………   Sites affected: ……………………

5.2.1.4 Measurement of antibody responses

D. farinae-specific total IgG and *D. farinae*-specific IgG subclasses were detected using the methods validated in Chapter 3 and Chapter 4 respectively. Western blotting was performed according to the methods described in Chapter 2.

5.2.1.5 Statistical analysis

Numerical data derived from the image analysis software were exported to Graphpad Prism, Version 3.0 for Windows (Graphpad software, San Diego, USA). As antibody concentrations in dogs are not normally distributed, the Kruskal-Wallis non-parametric ANOVA was used to compare antibody responses at multiple time points. If significant differences were detected by Kruskal-Wallis, comparisons between two individual time points were made by the Mann-Whitney test. A repeated measures test (Friedman test) was not possible due to the missing data points. The following comparisons were made: data from four time points (day 0, 90, 180 and 270) when all the dogs were combined as a single group; data from corresponding time points when dogs were divided into two groups based on those that showed no response to ASIT versus dogs that showed some response (complete or partial); data from corresponding time points when dogs were divided into three groups comprising those that showed a complete, partial or no response to ASIT; data from the four time points within each response group; and data from eight time points when dogs were divided into two groups based on those that received the standard ASIT protocol versus those that received the low-dose ASIT protocol. A p value of < 0.05 was considered to be significant.

5.2.2 Results

All the dogs had detectable levels of *D. farinae*-specific total IgG, IgG1 and IgG4 prior to the start of, and during, ASIT (Figure 5.1). Both total IgG, and the subclasses IgG1 and IgG4, recognised multiple proteins from *D. farinae* including 98- and 44-kDa major allergens and a 66-kDa minor allergen (Nuttall and others 2001b) (Figure 5.1).

Visual analysis of the strips from individual dogs showed that the strength of the bands varied during ASIT. However, there was no clearly obviously trend for the band intensity to increase or decrease with time. Over the course of 9 months of ASIT, individual dogs could show an obvious increase in band strength (Figure 5.1 a), a decrease (Figure. 5.1 b), remain relatively static (Figure 5.1 c), or fluctuate. For each dog, the changes seen with total *D. farinae*-specific IgG were approximately mirrored by similar changes in IgG1 and IgG4. No consistent induction or disappearance of specific bands at any molecular weight was observed.

This visual impression was confirmed by the numerical data from the image analysis software which showed that, during ASIT, there was no consistent increase in the total antibody response (the sum of all the band intensities on each strip) for IgG, IgG1 or IgG4 (Figure 5.2 a). There was also no consistent change in the response to the 98-kDa band (Figure 5.2 b) or the 44-kDa band (Figure 5.2 c) for any of the antibodies.

The statistical analysis of the data is summarised in Tables 5.4, 5.5 and 5.6. When all the dogs were considered as a single group, there was no significant difference in the total antibody response to *D. farinae* (sum of the band intensities on each strip) or the response to the 98-kDa and 44-kDa proteins at any of the four time points during ASIT for IgG, IgG1 or IgG4 ($p > 0.05$ for all nine analyses). However, when dogs that showed no response to ASIT were compared to dogs that showed a response (partial and complete response groups combined), there was a significant difference among the time points for total IgG. This difference was attributable to a significantly greater total IgG response to *D. farinae*, and the 98-kDa and 44-kDa proteins, in the group that was non-responsive to ASIT at day 0, compared to the response group at day 0 (Tables 5.4, 5.5 and 5.6). In the case of the 44-kDa band, there was also a significantly greater total IgG response in the non-responsive group at day 90 compared to the response group at day 90 (Table 5.6).

When the dogs were divided into three response groups and compared separately, there was a significant difference among time points for total IgG responses to *D.*

farinae (Table 5.4). This was due to a significantly higher IgG response in the non-responsive group at day 0, compared to the partial ($p = 0.019$) and the complete ($p = 0.011$) response groups. The total IgG responses to the 98-kDa and 44-kDa proteins were also significantly higher at day 0 in the non-response group compared with the complete response groups ($p = 0.023$ and $p = 0.042$, respectively) (Tables 5.4 and 5.5).

In addition, there was also significant variation among the time points in the standard- and low-dose ASIT protocols for IgG1 against total *D. farinae* antigens. This was attributable to significantly lower IgG1 levels to *D. farinae* at day 0 in the dogs receiving the low-dose protocol compared with those receiving the standard dose ($p = 0.01$) (Table 5.4). There were no other significant differences for *D. farinae* or the 98-kDa or 44-kDa proteins at any time point for any other antibody between the standard-dose and low-dose protocol groups.

Figure 5.1 Examples of immunoblots showing total IgG-, IgG1- and IgG4-binding profiles to *Dermatophagoides farinae* extract in dogs undergoing allergen-specific immunotherapy using alum-precipitated vaccines

Serum samples were collected before and after immunotherapy for 90, 180 and 270 days. The IgG responses to *D. farinae* allergens varied widely between dogs and could increase (a), decrease (b), remain the same (c) or fluctuate. Changes in total *D. farinae*-specific IgG were usually paralleled by equivalent changes in IgG1 and IgG4. MW, molecular weight in kilo Daltons (kDa).

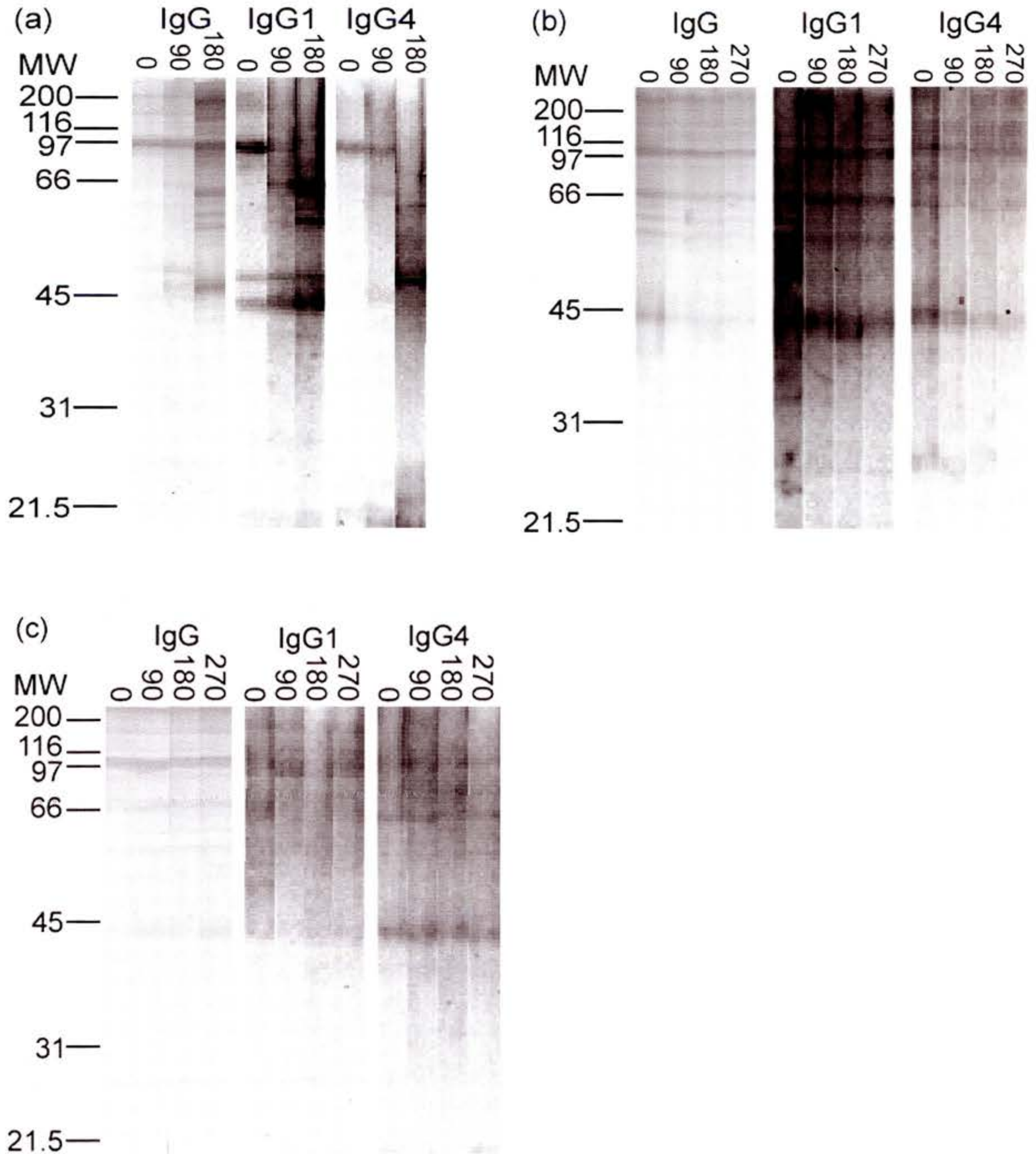


Figure 5.2 *Dermatophagoides farinae*-specific total IgG, IgG1 and IgG4 antibody responses during 9 months of allergen-specific immunotherapy using alum-precipitated vaccines

(a) The total antibody response to *D. farinae antigens* (calculated as the sum of all band intensities on each immunoblot strip). The dogs are divided into three groups categorized by clinical outcome. Dogs that received the standard-dose immunotherapy protocol are plotted in black. Dogs that received the low-dose immunotherapy protocol are plotted in red.

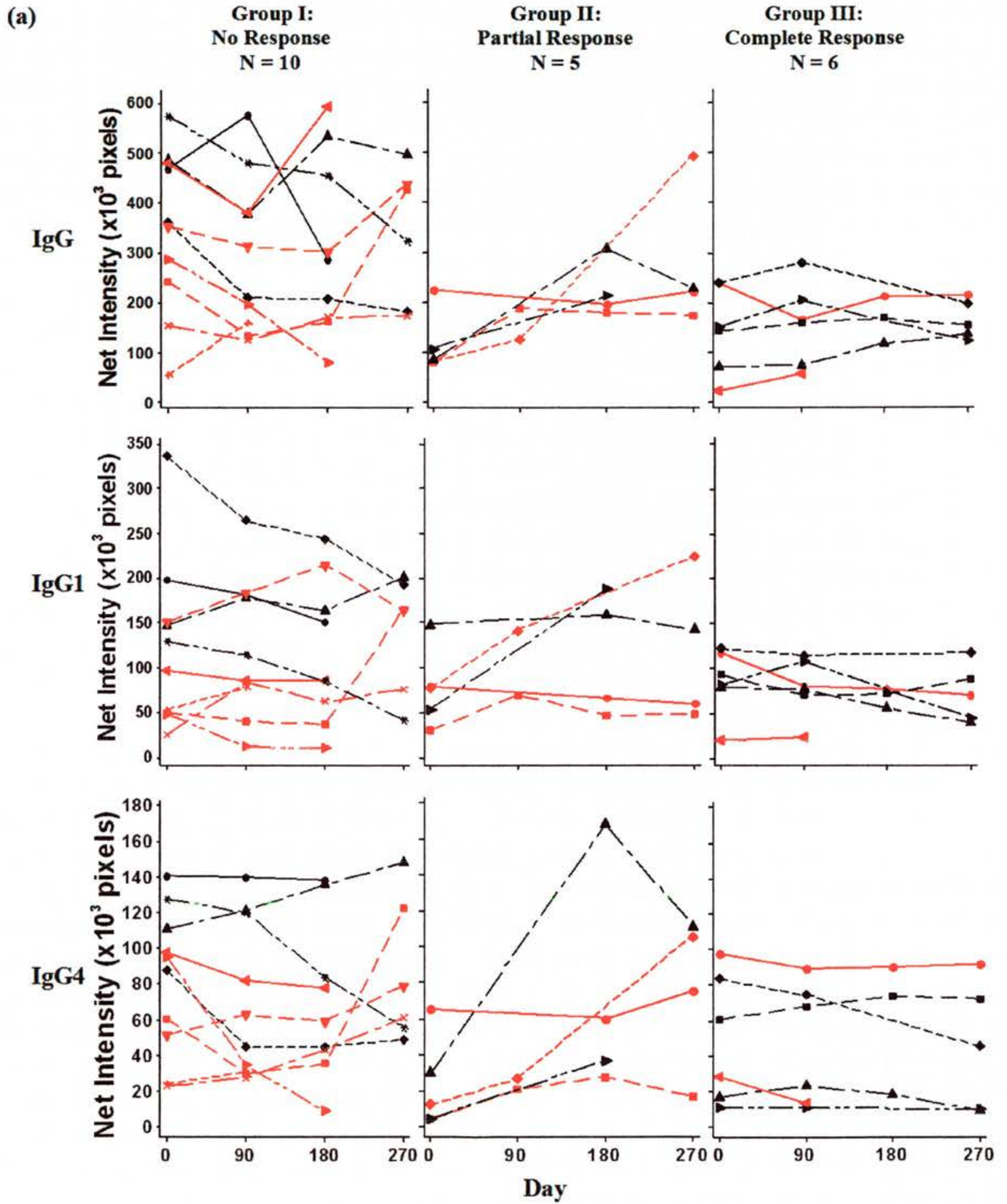


Figure 5.2 contd. *Dermatophagoides farinae*-specific total IgG, IgG1 and IgG4 antibody responses during 9 months of allergen-specific immunotherapy using alum-precipitated vaccine.

(b) Antibody responses to the 98-kDa band. The dogs are divided into three groups categorized by clinical outcome.

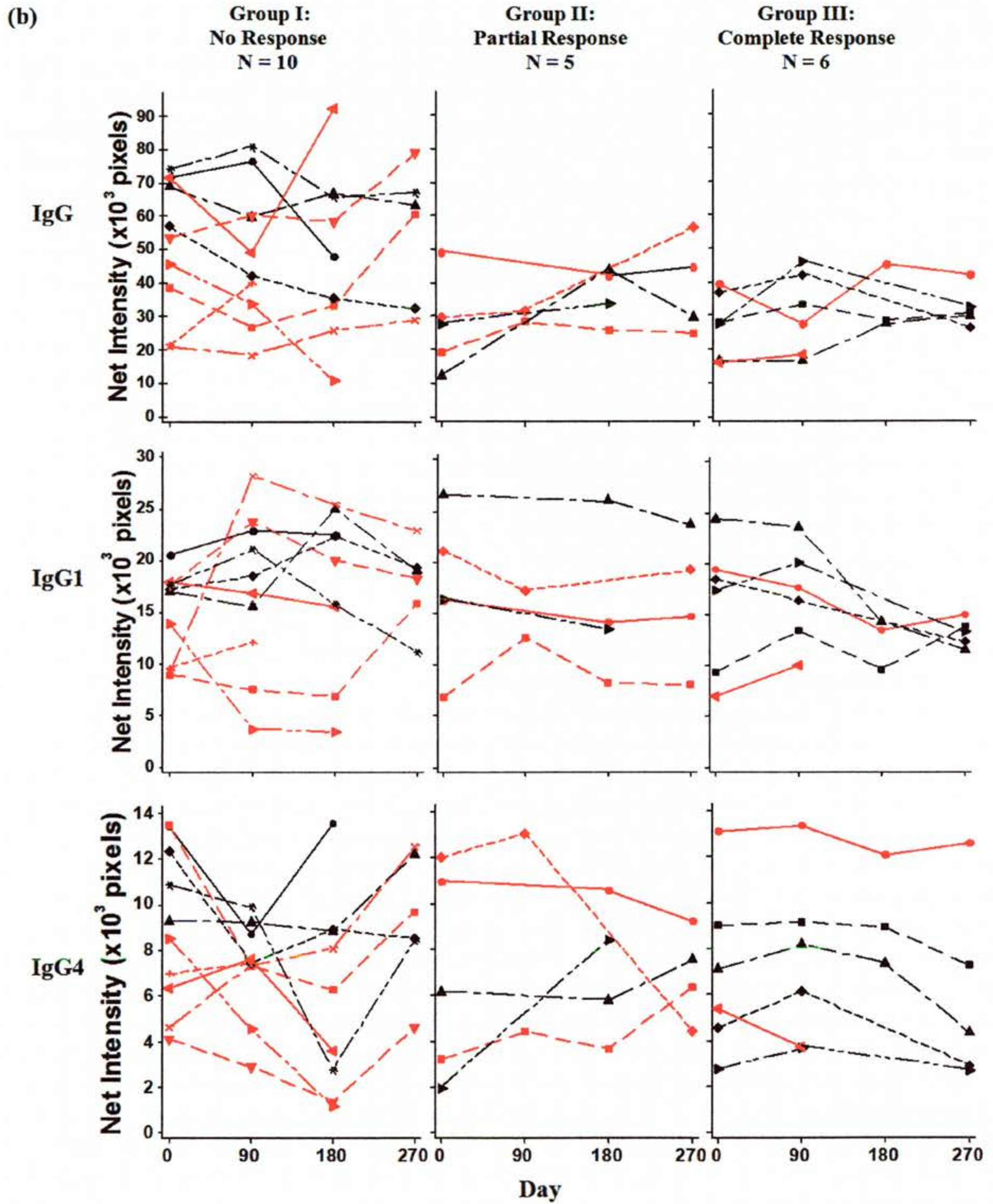


Figure 5.2 contd. ***Dermatophagoides farinae*-specific total IgG, IgG1 and IgG4 antibody responses during 9 months of allergen-specific immunotherapy using alum-precipitated vaccine.**

(c) Antibody responses to the 44-kDa band. The dogs are divided into three groups categorized by clinical outcome

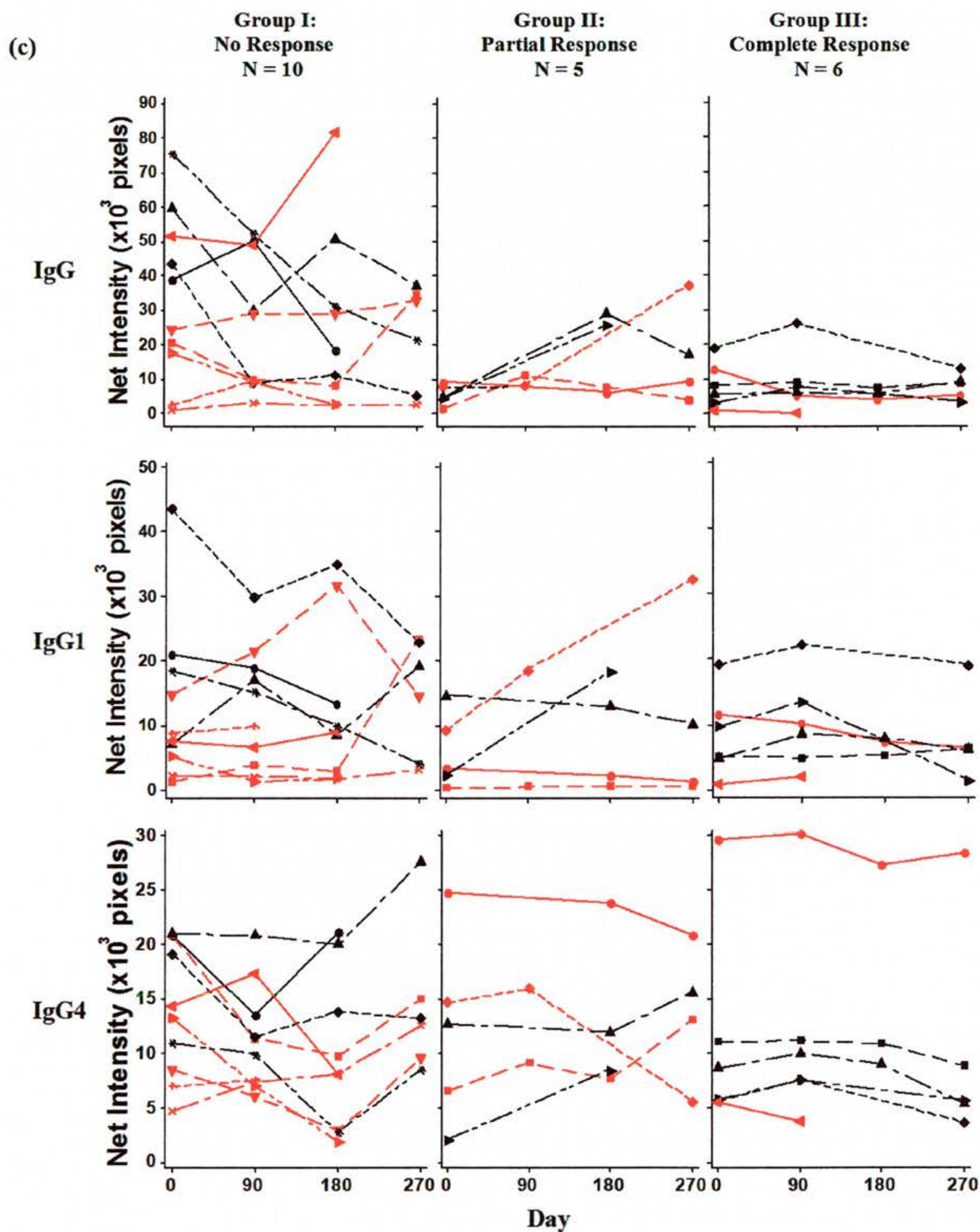


Table 5.4 Analysis of total antibody responses to *D. farinae* (sum of band intensities on each strip) during ASIT using alum-precipitated vaccine

Significant differences are shown in bold.

Antibody	Comparison	Time points analysed (days)	*p* value	Test
Total IgG	All dogs combined	0, 90, 180, 270	0.702	Kruskal Wallis
	No response group and response group	**N0, N90, N180, N270, R0, R90, R180, R270**	**0.006**	**Kruskal Wallis**
	No response group vs. response group	**N0 vs. R0**	**0.004**	**Mann Whitney**
	No response group vs. response group	N90 vs. R90	0.055	Mann Whitney
	No response group vs. response group	N180 vs. R180	0.408	Mann Whitney
	No response group vs. response group	N270 vs. R270	0.114	Mann Whitney
	The three response groups	**N0, P0, C0**	**0.012**	**Kruskal Wallis**
	No response vs. partial response	**N0 vs. P0**	**0.019**	**Mann Whitney**
	No response vs. complete response	**N0 vs. C0**	**0.011**	**Mann Whitney**
	Partial response vs. complete response	P0 vs. C0	0.662	Mann Whitney
	The three response groups	N90, P90, C90	0.148	Kruskal Wallis
	The three response groups	N180, P180, C180	0.364	Kruskal Wallis
	The three response groups	N270, P270, C270	0.062	Kruskal Wallis
	Complete response group	0, 90, 180, 270	0.978	Kruskal Wallis
	Partial response group	0, 90, 180, 270	0.095	Kruskal Wallis
	No response group	0, 90, 180, 270	0.869	Kruskal Wallis
	Standard dose versus low dose ASIT protocol	S0, S90, S180, S270, L0, L90, L180, L270	0.542	Kruskal Wallis

Table 5.4 contd. Analysis of total antibody responses to *D. farinae* (sum of band intensities on each strip) during ASIT using alum-precipitated vaccine

Significant differences are shown in bold.

Antibody	Comparison	Time points analysed (days)	*p* value	Test
IgG1	All dogs combined	0, 90, 180, 270	0.972	Kruskal Wallis
	No response group and response group	N0, N90, N180, N270, R0, R90, R180, R270	0.732	Kruskal Wallis
	The three response groups	N0, P0, C0	0.607	Kruskal Wallis
	The three response groups	N90, P90, C90	0.541	Kruskal Wallis
	The three response groups	N180, P180, C180	0.677	Kruskal Wallis
	The three response groups	N270, P270, C270	0.253	Kruskal Wallis
	Complete response group	0, 90, 180, 270	0.551	Kruskal Wallis
	Partial response group	0, 90, 180, 270	0.908	Kruskal Wallis
	No response group	0, 90, 180, 270	0.895	Kruskal Wallis
	Standard dose versus low dose ASIT protocol	**S0, S90, S180, S270, L0, L90, L180, L270**	**0.037**	**Kruskal Wallis**
	Standard dose versus low dose ASIT protocol	**S0 vs. L0**	**0.010**	**Mann Whitney**
	Standard dose versus low dose ASIT protocol	S90 vs. L90	0.068	Mann Whitney
	Standard dose versus low dose ASIT protocol	S180 vs. L180	0.065	Mann Whitney
	Standard dose versus low dose ASIT protocol	S270 vs. L270	0.694	Mann Whitney

Table 5.4 contd. Analysis of total antibody responses to *D. farinae* (sum of band intensities on each strip) during ASIT using alum-precipitated vaccine

Significant differences are shown in bold.

Antibody	Comparison	Time points analysed (days)	*p* value	Test
IgG4	All dogs combined	0, 90, 180, 270	0.716	Kruskal Wallis
	No response group and response group	N0, N90, N180, N270, R0, R90, R180, R270	0.232	Kruskal Wallis
	The three response groups	N0, P0, C0	0.071	Kruskal Wallis
	The three response groups	N90, P90, C90	0.308	Kruskal Wallis
	The three response groups	N180, P180, C180	0.962	Kruskal Wallis
	The three response groups	N270, P270, C270	0.340	Kruskal Wallis
	Complete response group	0, 90, 180, 270	0.907	Kruskal Wallis
	Partial response group	0, 90, 180, 270	0.225	Kruskal Wallis
	No response group	0, 90, 180, 270	0.817	Kruskal Wallis
	Standard dose versus low dose ASIT protocol	S0, S90, S180, S270, L0, L90, L180, L270	0.781	Kruskal Wallis

C: dogs showing a complete response to ASIT; L: low dose ASIT protocol; N: no-response group; P: dogs showing a partial response to ASIT; R: dogs that responded to ASIT (partial or complete); S: standard dose ASIT protocol.

Table 5.5 Analysis of antibody responses to the 98-kDa band from *D. farinae* during ASIT using alum-precipitated vaccine

Significant differences are shown in bold.

Antibody	Comparison	Time points analysed (days)	*p* value	Test
Total IgG	All dogs combined	0, 90, 180, 270	0.873	Kruskal Wallis
	No response group and response group	**N0, N90, N180, N270, R0, R90, R180, R270**	**0.013**	**Kruskal Wallis**
	No response group vs. response group	**N0 vs. R0**	**0.008**	**Mann Whitney**
	No response group vs. response group	N90 vs. R90	0.055	Mann Whitney
	No response group vs. response group	N180 vs. R180	0.299	Mann Whitney
	No response group vs. response group	N270 vs. R270	0.066	Mann Whitney
	The three response groups	**N0, P0, C0**	**0.028**	**Kruskal Wallis**
	No response vs. partial response	**N0 vs. P0**	**0.040**	**Mann Whitney**
	No response vs. complete response	**N0 vs. C0**	**0.023**	**Mann Whitney**
	Partial response vs. complete response	P0 vs. C0	1.000	Mann Whitney
	The three response groups	N90, P90, C90	0.148	Kruskal Wallis
	The three response groups	N180, P180, C180	0.532	Kruskal Wallis
	The three response groups	N270, P270, C270	0.161	Kruskal Wallis
	Complete response group	0, 90, 180, 270	0.739	Kruskal Wallis
	Partial response group	0, 90, 180, 270	0.540	Kruskal Wallis
	No response group	0, 90, 180, 270	0.880	Kruskal Wallis
	Standard dose versus low dose ASIT protocol	S0, S90, S180, S270, L0, L90, L180, L270	0.630	Kruskal Wallis

Table 5.5 contd. Analysis of antibody responses to the 98-kDa band from *D. farinae* during ASIT using alum-precipitated vaccine

Significant differences are shown in bold.

Antibody	Comparison	Time points analysed (days)	*p* value	Test
IgG1	All dogs combined	0, 90, 180, 270	0.961	Kruskal Wallis
	No response group and response group	N0, N90, N180, N270, R0, R90, R180, R270	0.711	Kruskal Wallis
	The three response groups	N0, P0, C0	0.813	Kruskal Wallis
	The three response groups	N90, P90, C90	0.845	Kruskal Wallis
	The three response groups	N180, P180, C180	0.439	Kruskal Wallis
	The three response groups	N270, P270, C270	0.214	Kruskal Wallis
	Complete response group	0, 90, 180, 270	0.432	Kruskal Wallis
	Partial response group	0, 90, 180, 270	0.540	Kruskal Wallis
	No response group	0, 90, 180, 270	0.905	Kruskal Wallis
	Standard dose versus low dose ASIT protocol	S0, S90, S180, S270, L0, L90, L180, L270	0.220	Kruskal Wallis

Table 5.5 contd. Analysis of antibody responses to the 98-kDa band from *D. farinae* during ASIT using alum-precipitated vaccine

Significant differences are shown in bold.

Antibody	Comparison	Time points analysed (days)	*p* value	Test
IgG4	All dogs combined	0, 90, 180, 270	0.967	Kruskal Wallis
	No response group and response group	N0, N90, N180, N270, R0, R90, R180, R270	0.728	Kruskal Wallis
	The three response groups	N0, P0, C0	0.536	Kruskal Wallis
	The three response groups	N90, P90, C90	0.829	Kruskal Wallis
	The three response groups	N180, P180, C180	0.405	Kruskal Wallis
	The three response groups	N270, P270, C270	0.340	Kruskal Wallis
	Complete response group	0, 90, 180, 270	0.482	Kruskal Wallis
	Partial response group	0, 90, 180, 270	0.950	Kruskal Wallis
	No response group	0, 90, 180, 270	0.403	Kruskal Wallis
	Standard dose versus low dose ASIT protocol	S0, S90, S180, S270, L0, L90, L180, L270	0.891	Kruskal Wallis

C: dogs showing a complete response to ASIT; L: low dose ASIT protocol; N: no-response group; P: dogs showing a partial response to ASIT; R: dogs that responded to ASIT (partial or complete); S: standard dose ASIT protocol.

Table 5.6 Analysis of antibody responses to the 44-kDa band from *D. farinae* during ASIT using alum-precipitated vaccine

Significant differences are shown in bold.

Antibody	Comparison	Time points analysed (days)	*p* value	Test
Total IgG	All dogs combined	0, 90, 180, 270	0.975	Kruskal Wallis
	No response group and response group	**N0, N90, N180, N270, R0, R90, R180, R270**	**0.046**	**Kruskal Wallis**
	No response group vs. response group	**N0 vs. R0**	**0.015**	**Mann Whitney**
	No response group vs. response group	**N90 vs. R90**	**0.043**	**Mann Whitney**
	No response group vs. response group	N180 vs. R180	0.252	Mann Whitney
	No response group vs. response group	N270 vs. R270	0.328	Mann Whitney
	The three response groups	**N0, P0, C0**	**0.046**	**Kruskal Wallis**
	No response vs. partial response	N0 vs. P0	0.055	Mann Whitney
	No response vs. complete response	**N0 vs. C0**	**0.042**	**Mann Whitney**
	Partial response vs. complete response	P0 vs. C0	0.662	Mann Whitney
	The three response groups	N90, P90, C90	0.095	Kruskal Wallis
	The three response groups	N180, P180, C180	0.228	Kruskal Wallis
	The three response groups	N270, P270, C270	0.328	Kruskal Wallis
	Complete response group	0, 90, 180, 270	0.889	Kruskal Wallis
	Partial response group	0, 90, 180, 270	0.227	Kruskal Wallis
	No response group	0, 90, 180, 270	0.872	Kruskal Wallis
	Standard dose versus low dose ASIT protocol	S0, S90, S180, S270, L0, L90, L180, L270	0.626	Kruskal Wallis

Table 5.6 contd. Analysis of antibody responses to the 44-kDa band from *D. farinae* during ASIT using alum-precipitated vaccine

Significant differences are shown in bold.

Antibody	Comparison	Time points analysed (days)	*p* value	Test
IgG1	All dogs combined	0, 90, 180, 270	0.909	Kruskal Wallis
	No response group and response group	N0, N90, N180, N270, R0, R90, R180, R270	0.719	Kruskal Wallis
	The three response groups	N0, P0, C0	0.448	Kruskal Wallis
	The three response groups	N90, P90, C90	0.806	Kruskal Wallis
	The three response groups	N180, P180, C180	0.714	Kruskal Wallis
	The three response groups	N270, P270, C270	0.498	Kruskal Wallis
	Complete response group	0, 90, 180, 270	0.870	Kruskal Wallis
	Partial response group	0, 90, 180, 270	0.967	Kruskal Wallis
	No response group	0, 90, 180, 270	0.864	Kruskal Wallis
	Standard dose versus low dose ASIT protocol	S0, S90, S180, S270, L0, L90, L180, L270	0.065	Kruskal Wallis

Table 5.6 contd. Analysis of antibody responses to the 44-kDa band from *D. farinae* during ASIT using alum-precipitated vaccine

Significant differences are shown in bold.

Antibody	Comparison	Time points analysed (days)	*p* value	Test
IgG4	All dogs combined	0, 90, 180, 270	0.967	Kruskal Wallis
	No response group and response group	N0, N90, N180, N270, R0, R90, R180, R270	0.728	Kruskal Wallis
	The three response groups	N0, P0, C0	0.536	Kruskal Wallis
	The three response groups	N90, P90, C90	0.829	Kruskal Wallis
	The three response groups	N180, P180, C180	0.405	Kruskal Wallis
	The three response groups	N270, P270, C270	0.340	Kruskal Wallis
	Complete response group	0, 90, 180, 270	0.482	Kruskal Wallis
	Partial response group	0, 90, 180, 270	0.950	Kruskal Wallis
	No response group	0, 90, 180, 270	0.403	Kruskal Wallis
	Standard dose versus low dose ASIT protocol	S0, S90, S180, S270, L0, L90, L180, L270	0.891	Kruskal Wallis

C: dogs showing a complete response to ASIT; L: low dose ASIT protocol; N: no-response group; P: dogs showing a partial response to ASIT; R: dogs that responded to ASIT (partial or complete); S: standard dose ASIT protocol.

5.2.3 Discussion

During ASIT with alum-precipitated vaccines, there was no consistent augmentation of the total quantity of *D. farinae*-specific IgG or IgG subclass antibodies to antigens from *D. farinae*. Levels of the antibodies could increase or fall but there was no significant increase by day 270 compared to day 0. This was the case for the total response to all the *D. farinae* proteins (measured by summing all the band intensities on each strip) as well as the response to the 98-kD and 44-kDa proteins. These latter proteins were chosen for specific analysis because they were the strongest and most visually obvious bands on the strips.

Although not exactly parallel, the changes in IgG1 and IgG4 tended to mirror the changes seen with total IgG. The lack of significant increases in levels of allergen-specific IgG, IgG1 and IgG4 is surprising. Previous studies have demonstrated that increases in allergen-specific IgG1 and IgG4 are seen following ASIT in man (Muller and others 1989; McHugh and others 1990; van Neerven and others 1999). An increase in total (non-allergen-specific) IgG1 was also observed in most dogs that were given ASIT using alum-precipitated vaccines (Fraser and others 2004). The reason for the difference in these findings between our study and previous studies is not clear, but there are a number of possible explanations. First, although administration of adjuvanted proteins should lead to a marked IgG response, some studies in humans have shown poor clinical efficacy to alum-precipitated vaccines as well as a lack of alteration in immune responses (Lichtenstein and others 1968; Bousquet and others 1985). It is possible that the vaccines used in this study did not contain sufficient protein to induce an allergen-specific IgG response, even though they were shown to elicit an increase in total IgG1 in most of the dogs in a prior study (Fraser and others 2004). The allergen manufacturer used in this study does not provide the specific protein content of allergens within vaccines, so it is not possible to know the precise amount of *D. farinae* that is present. Further, the quantity of *D. farinae* in each vaccine is likely to be different depending on the number of other allergens included. It is also possible that the amount of *D. farinae* antigens may vary from batch to batch, leading to further variation in specific immune responses. Evidence for the above possibility is provided by the lack of

significant differences in IgG, IgG1 and IgG4 levels in dogs receiving either the standard ASIT protocol or the low-dose protocol, which involved administering 1/10 of the recommended dose. It would have been expected that dogs receiving the higher doses would have mounted a greater antibody response, as long as sufficient protein was present initially. It is also important that allergen manufacturers work towards standardisation of allergens so that the precise protein concentrations can be included in vaccines, allowing direct comparison.

A second possibility for the discrepancy may relate to the different methods employed in the various studies. In this study we used a validated semi-quantitative blot analysis system in which the net intensity of bands shows a linear relationship to log antibody concentration. This system allows a whole range of separated proteins from *D. farinae* to be studied simultaneously. This is a major advantage compared with ELISA assays, which can only measure a single protein at a time. However, ELISA assays can provide a more accurate measurement of a protein's concentration in mg/ml and they tend to have lower coefficients of variation, indicating superior repeatability. Hence, in future studies, a direct comparison of the results obtained by blot analysis and ELISA warrants investigation.

Thirdly, the increase in total IgG documented in the earlier study (Fraser and others 2004) was quantified using a radial-immuno-diffusion technique. This method measured the total quantity of IgG1 present and did not relate to specific allergens. Hence, the increase seen in that study could have been due to other allergens or non-specific IgG activation and not directly related to *D. farinae* proteins.

Finally, it is possible that production of *D. farinae* specific IgG antibodies is not induced in dogs with AD undergoing ASIT. This has not previously been studied and it is not known whether the formulation of the allergens, or the expression of the epitopes of major allergens in ASIT, is appropriate or sufficient to stimulate this arm of the immune system. If not, clinical efficacy may be related to some other mechanism such as down-regulation of cellular function or changes in cytokine profile.

An interesting finding arising from this study was that dogs showing no response to ASIT had significantly higher *D. farinae*-specific total IgG levels on day 0 compared with dogs showing a partial or complete response. This was the case both for total *D. farinae* antigens, and the 98-kDa and 44-kDa proteins. This suggested that if a dog with AD had a pre-existing high level of IgG antibodies to *D. farinae* prior to the onset of ASIT, it was less likely to respond. This is a potentially exciting finding, as it may suggest a means by which dogs could be screened prior to initiation of ASIT. Dogs with a low level of IgG antibodies could be given a better prognosis than those with a high level. Alternatively, this might allow alterations in dosage schedule to be predicted in advance. This finding clearly needs to be substantiated using additional methods and in a larger group of dogs to ensure that it is a repeatable phenomenon. If so, the discriminatory power of the measurement would have to be carefully determined.

A statistical quirk that arose in this study was the finding that dogs receiving the standard-dose ASIT protocol had significantly higher levels of IgG1 to total *D. farinae* proteins at day 0 compared with those receiving the low-dose protocol. This can only have arisen by chance, as the dogs were assigned to their relative groups using block randomisation. This chance occurrence did not seem to have any bearing on the subsequent efficacy of the ASIT because there were no significant differences in the response rates between the two groups (Colombo and others 2005).

In summary, this study did not demonstrate the production of *D. farinae*-specific IgG blocking antibodies in atopic dogs undergoing ASIT based on alum-precipitated vaccines. However, a high pre-existing level of total IgG to *D. farinae* antigens, including the 98-kDa and 44-kDa proteins, suggested that dogs were less likely to respond to ASIT.

5.3 Study 2 – ASIT using aqueous vaccines

As a follow-on to the ASIT study using alum-precipitated vaccines, a pilot study was performed to determine if aqueous vaccines might elicit IgG responses. As this form of vaccine was not used at the host institution, samples had to be obtained from the USA. Also, the study design was necessarily different because all aspects of the case inclusion, monitoring and follow-up could not be controlled.

5.3.1 Material and methods

5.3.1.1 Serum samples

Serum samples from 15 atopic dogs were recruited from the Animal Dermatology Clinic, San Diego, California, USA. All 15 dogs included had a positive reaction to *D. farinae* at a dilution of 1/1000 w/v in an intradermal test. Samples were collected before the start of ASIT and after 2 to 7.5 months of treatment. The variation in timing of the post ASIT sample was due to logistical limitations within the study population. However, as the aim was to determine whether or not there was an increase in *D. farinae* specific IgG, and not the magnitude of that response, this was not considered to be a problem. After collection, serum was separated by centrifugation and stored at -20°C until use.

5.3.1.2 Immunotherapy protocol

The content of ASIT vaccines was determined based on correlation of the positive skin test results with the dog's history. All vaccines were prepared with allergen extracts obtained from Greer Laboratories (Lenoir, NC, USA), and given subcutaneously. The ASIT protocols used are shown in Table 5.7. The number of allergens in the treatment sets varied from 6 to 12. All allergens were mixed from stock solutions that contained between 10,000 and 20,000 protein nitrogen units (PNU)/ml. *Dermatophagoides farinae* stock solution contained 10,000 PNU/ml. All dogs underwent an induction phase that consisted of two vials of allergens. Vial one contained a total of 1,000 – 1,800 PNU/ml and vial two of 10,000 – 18,000 PNU/ml,

depending on allergen content. The concentration of *Dermatophagoides farinae* varied from 83 PNU/ml to 167 PNU/ml in vial 1 and from 833 PNU/ml to 1,667 PNU/m in vial 2. These ASIT schedules were followed unless the dog had an adverse reaction to an allergen injection, which most commonly consisted of increased pruritus following injection. If this occurred the volume of allergen injected was decreased. Final maintenance doses varied from 0.5ml to 1.0ml and were given at intervals of 5 to 20 days, depending on the patient's response.

Table 5.7 Allergen-specific immunotherapy protocols used in the aqueous vaccines study

		Dog under 20lbs	Dog over 20lbs
Vial	Day	Volume (ml)	Volume (ml)
1	1	0.1	0.1
	3	0.2	0.2
	5	0.3	0.3
	7	0.4	0.4
	9	0.6	0.6
	11	0.8	0.8
	13	1.0	1.0
2	15	0.1	0.2
	17	0.2	0.3
	19	0.3	0.4
	21	0.5	0.6
	23	0.5	0.8
	25	0.5	1.0
	30	0.5	
	35	0.5	1.0
	40	0.7	
	45		1.0
	50	0.7	
	55		1.0
	60	0.7	
	69		1.0
	70	0.7	
	80	0.7	
	83		1.0
	94	0.7	
	97		1.0
	108	0.7	
	111		1.0

5.3.1.3 Clinical assessment

In this study, dogs were assessed by means of a pruritus visual analogue scale that was currently in use at the practice concerned (Figure 5.3).

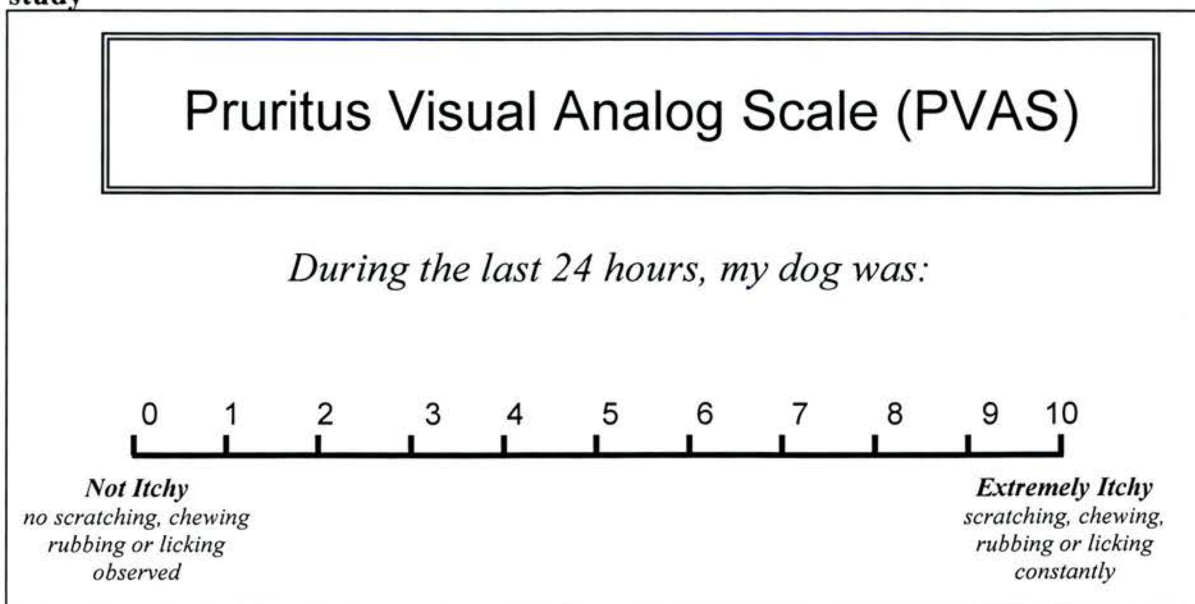
5.3.1.4 Measurement of antibody responses

D. farinae-specific total IgG was detected using the methods validated in Chapter 3. Due to insufficient quantities of reagents, IgG1 and IgG4 responses could not be evaluated in this study. Western blotting was performed according to the methods described in Chapter 2.

5.3.1.5 Statistical analysis

Numerical data derived from the image analysis software were exported to GraphPad Prism, Version 3.0 for Windows (GraphPad software, San Diego, USA). As in the alum-precipitated study, results were analysed using a non-parametric test. As only two time points were available for this study, the Wilcoxon matched-pair signed-ranks test was used to compare antibody responses before and after ASIT. A p value of < 0.05 was considered to be significant.

Figure 5.3 Pruritus visual analogue scale used in the aqueous vaccine study



5.3.2 Results

As in the alum-precipitated vaccine study, all the atopic dogs included in the study had detectable levels of *D. farinae*-specific total IgG prior to the start of, and after, ASIT (Fig. 5.3). Multiple proteins from *D. farinae* were recognised, and the most visually obvious bands had molecular weights of approximately 98 kDa and 44 kDa (Fig. 5.4). Visual analysis of the strips from individual dogs showed that the strength of certain individual bands increased after ASIT, and new bands became visible (Fig. 5.4).

This visual impression was confirmed by the numerical data from the image analysis software which showed that, during ASIT, there was a significant increase in the total IgG response (the sum of all the band intensities on each strip) to *D. farinae* antigens (p=0.015, Fig. 5.5 a). To facilitate further quantitative analysis, three molecular weight zones were chosen for statistical evaluation: bands with a MW of 98 kDa, bands with MWs between 50-70 kDa and bands with MWs between 30-45 kDa. These zones were selected because most of the visible bands fell within them.

There was a significant increase in the IgG response to the 98 kDa band (p=0.015, Fig. 5.5 b), the 50-70 kDa bands (p=0.012, Fig. 5.5 c) and the 30-45 kDa bands (p=0.035, Fig. 5.5 d).

The severity of pruritus as assessed by the visual analog scale (PVAS) is summarised in Table 5.8. There was a significant decrease in pruritus scale ($p<0.001$) after ASIT using aqueous vaccines (Fig 5.6). In order to correlate the *D. farinae*-specific IgG response with clinical improvement, percentage of increased IgG intensity was plotted against percentage of decreased PVAS score (Fig. 5.7). However, the R-square value ranged from 0.0537 to 0.1338 and no correlation was found between *D. farinae*-specific IgG level and pruritus score (Fig. 5.7).

Figure 5.4 Total IgG binding profile to *Dermatophagoides farinae* extracts detected by immunoblotting in dogs undergoing allergen-specific immunotherapy with aqueous vaccines

15 dogs were included. Serum samples were collected before (strip on the left) and after 2 – 7.5 months (strip on the right) of immunotherapy. In the majority of dogs, IgG responses to *D. farinae* allergens visibly increased after immunotherapy, either in terms of band intensity or number of bands. The molecular weight standards on the left are in kilo-dalton (kDa).

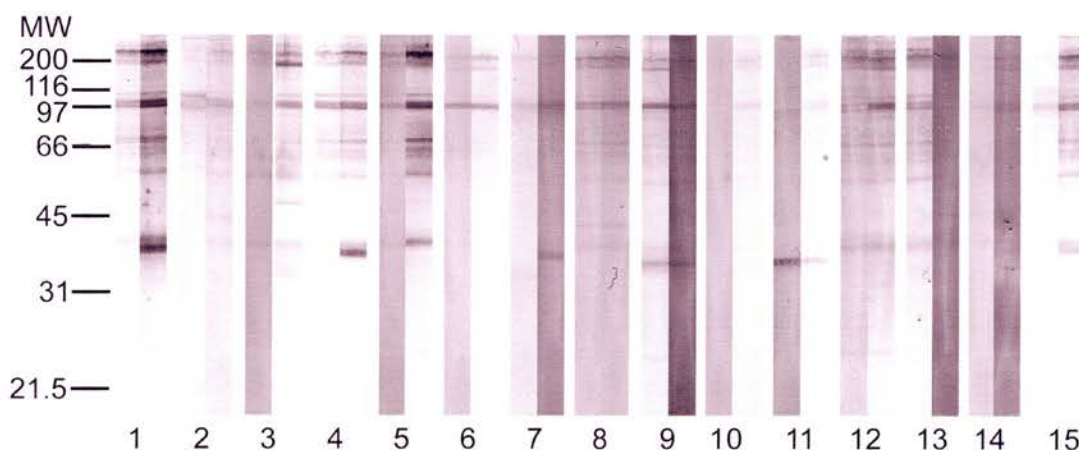


Figure 5.5 Significant increases in IgG responses to *D. farinae* proteins, measured by digital image analysis of bands on Western blots (shown as net intensity)

a: The total IgG response to all *D. farinae* antigens (the sum of all band intensities on each strip, p=0.015). b: The IgG response to a 98 kDa band (p=0.015). c: The IgG response to bands with molecular weights between 50-70 kDa (p=0.012). d: The IgG response to bands with molecular weights between 30-45 kDa (p=0.035).

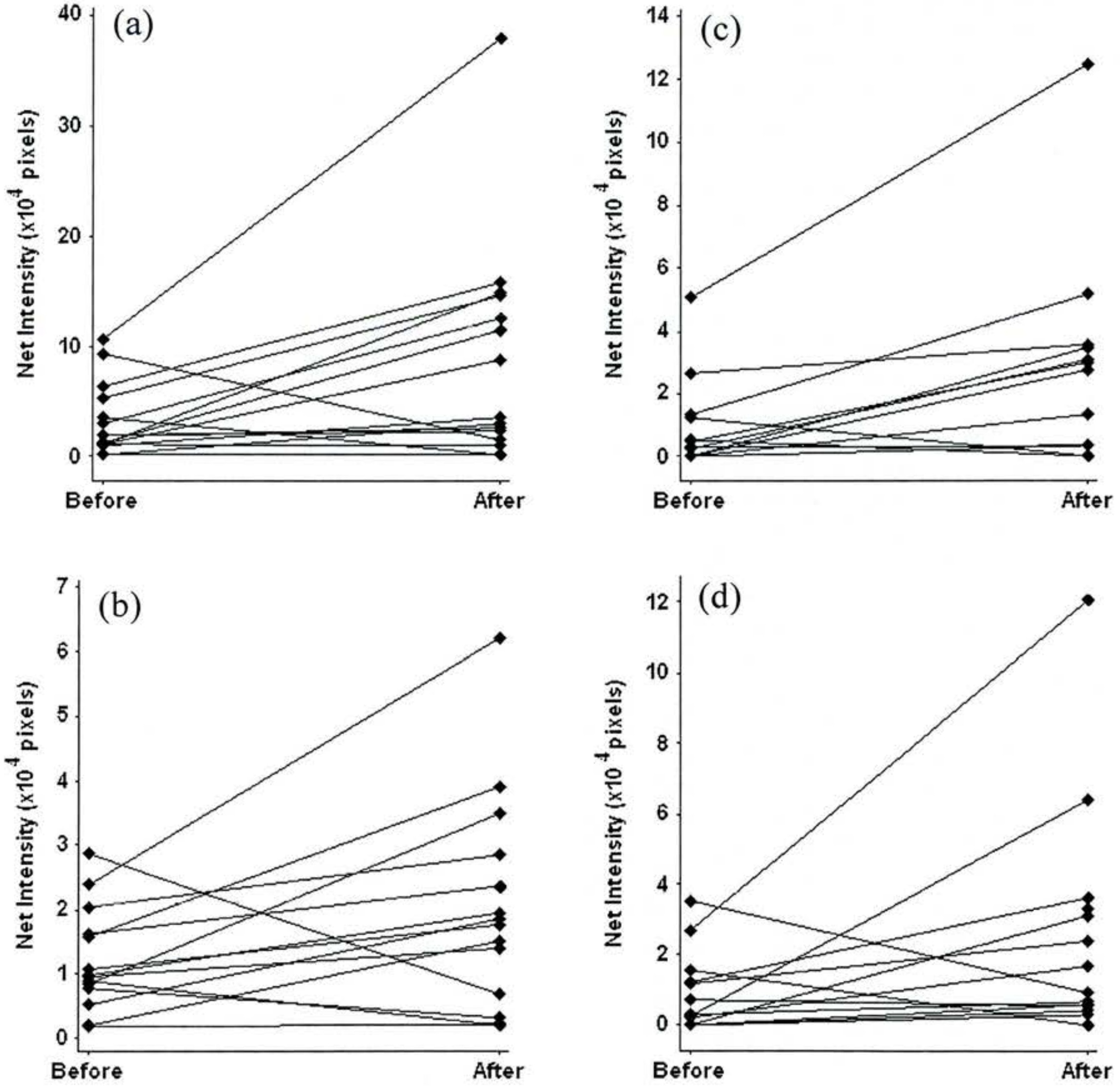


Table 5.8 Pruritus visual analog scale scores in dogs undergoing ASIT with aqueous vaccines

No	Breed	Sex	Age	Df Rx in IDT	PVAS Score	
					Pre-ASIT	Post-ASIT
1	Chow chow mix	MN	5	5	4	1
2	Australian cattle dog mix	MN	2	4	10	6
3	Labrador retriever	MN	3	4	6	2
4	Wirehaired pointing griffon	F	1.5	5	4	2
5	Jack Russell terrier	FS	10	4	9	1
6	Jack Russell terrier	MN	8	4	7	3
7	Border collie	F	1	5	7	4
8	Bichon Frise	FS	5	4	10	10
9	Mixed breed	FS	5	2	6	6
10	Boston terrier	FS	3	4	10	5
11	Lhasa Apso mix	MN	3	2-3	8	6
12	American pit bull terrier mix	M	5	4	7	4
13	German Shepherd	M	2	4	5	1
14	Leonberger	MN	3	4	6	2
15	Australian Shepherd mix	MN	1.5	4-5	7	8

MN: male-neutered; M: male-intact; FS: female-spayed; F: female-intact; PVAS: pruritus visual analog scale

Figure 5.6 Significant decrease in pruritus score after ASIT using aqueous vaccine

The pruritus severity in dogs assessed by a pruritus visual analogue scale is shown. The pruritus score significantly decreased after ASIT using aqueous vaccines (p=0.001). PVAS: pruritus visual analog scale.

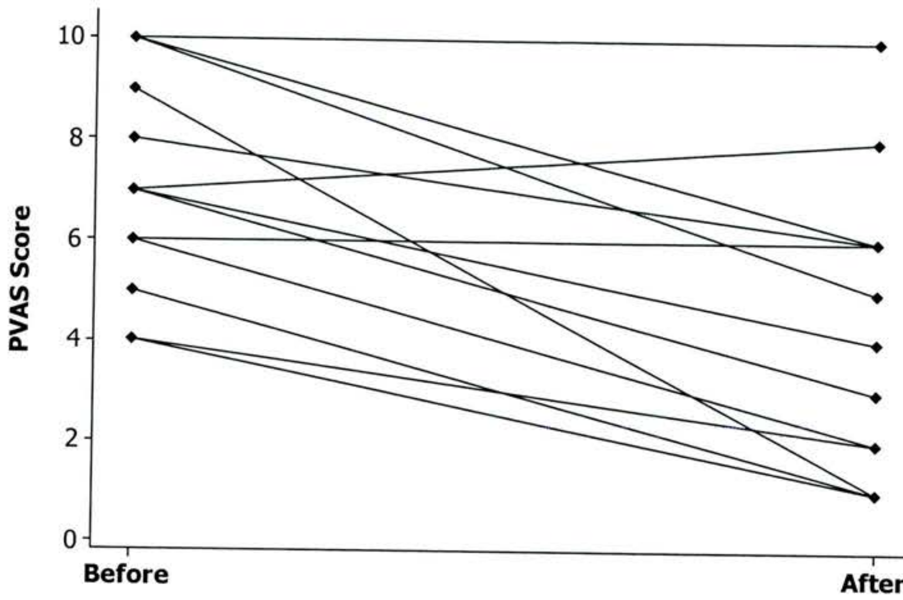
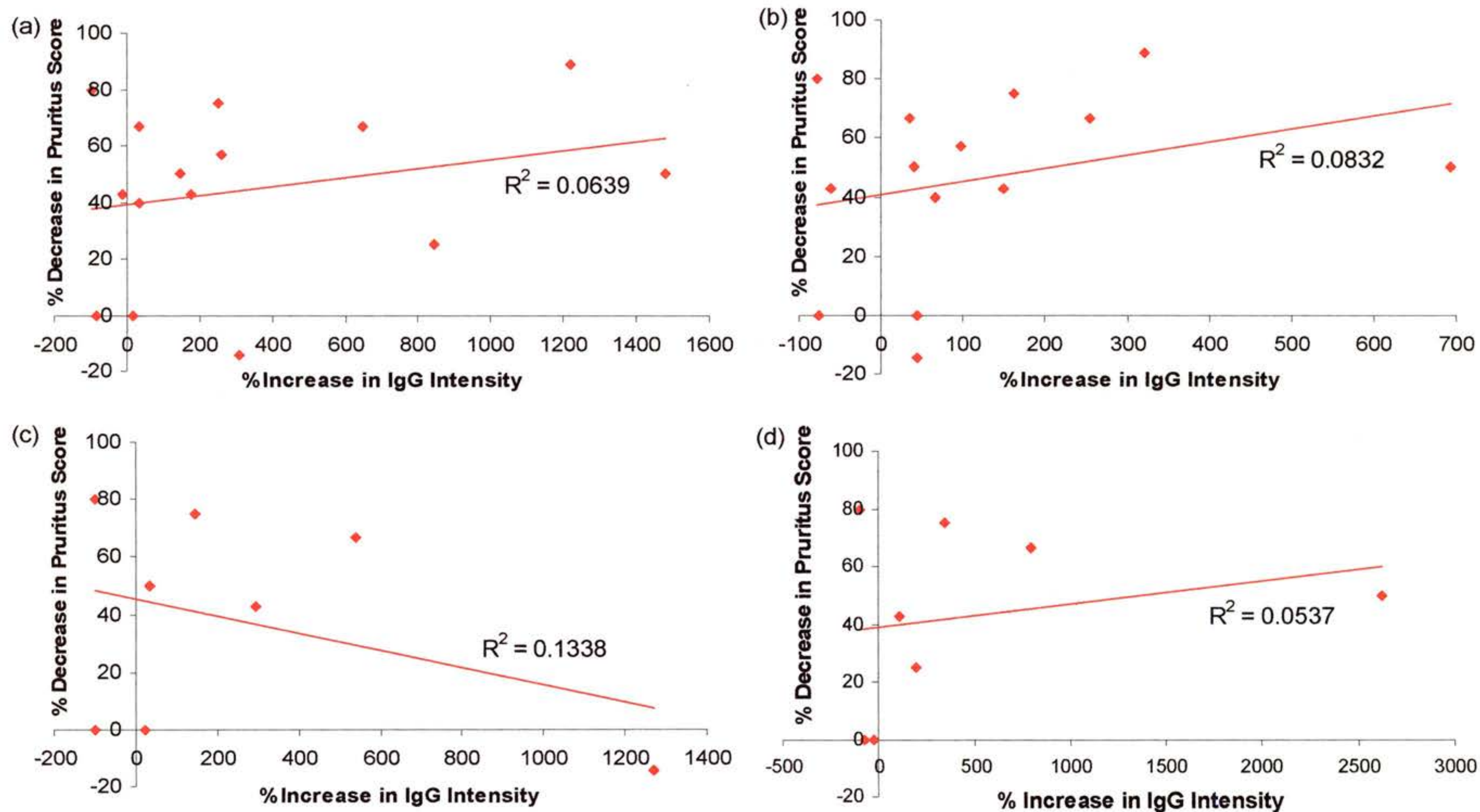


Figure 5.7 The relationship between increased specific IgG response and decreased pruritus score

No correlation was found between *D. farinae*-specific IgG response and pruritus score. a: total *D. farinae*-specific IgG; b: 98-kDa specific IgG; c: 50-70-kDa specific IgG; d: 30-45-kDa specific IgG.



5.3.3 Discussion

In this study, we documented allergen-specific total IgG responses to various antigens of the house dust mite *D. farinae* in dogs with AD. During ASIT with aqueous vaccines, there was a significant increase in the quantity of *D. farinae*-specific IgG antibodies to various antigens from the mite. This is in contrast to the results with alum-precipitated vaccines, which documented no significant increase in IgG concentrations when using exactly the same methodology. The finding that aqueous vaccines produced a measurable immune response that was not seen with alum-precipitated vaccines has important implications and raises two important questions. First, why were the alum-precipitated vaccines not found to elicit such IgG responses? This would clearly be expected when a foreign protein is injected into a dog. An increase in total (non-allergen-specific) IgG1 was observed in most dogs that were given ASIT using alum precipitated vaccines (Fraser and others 2004). This raises the possibility that alum-precipitated vaccines induce a non-specific augmentation of the IgG response, but do not induce specific antibodies against major allergens that are relevant in canine atopic dermatitis. Second, does the antigen-specific IgG response seen with aqueous vaccines represent "blocking antibodies", and if so, are these antibodies important in the mode of action of ASIT? If that was the case, it would be expected that the efficacy of aqueous vaccines in dogs would be superior to alum-precipitated vaccines. Further studies are clearly warranted to answer these specific questions.

The increase in IgG concentrations was seen to a number of proteins with differing molecular weights. This resulted in an overall increase in the total IgG response to *D. farinae* antigens, as well significant increases in the response to 98 kDa, 50-70 kDa and 30-45 kDa proteins. This finding provides some support for the hypothesis that ASIT induces blocking antibodies because the IgG and IgE would be competing for the same major allergens. It should be emphasised that the relationship between IgG concentrations and net intensity of the bands on the blots is a log relationship, and therefore a certain increase in net intensity represents an even greater increase in IgG concentration. The increase in IgG concentrations to lower molecular weight

antigens was very apparent in some dogs. This suggests that injection of some *D. farinae* proteins induces a specific IgG response that isn't seen in atopic dogs prior to the start of ASIT. Whether these antibodies are of any clinical relevance, or contribute to the efficacy of ASIT, remains to be determined.

The significant augmentation of specific antibody responses following ASIT with aqueous allergens is in accordance with earlier publications. In humans, previous studies have demonstrated that ASIT results in an increase in the serum concentration of allergen-specific IgG antibodies with specificity for the injected antigens (Yunginger 1987; Muller and others 1989; McHugh and others 1990). In atopic dogs, concentrations of pollen-specific IgG were shown to increase following at least 6-months of ASIT using aqueous allergens (Hites and others 1989). Our study is the first to demonstrate an increase in *D. farinae* specific IgG following ASIT.

It was not surprising that the correlation between the induced IgG responses and clinical outcome was poor because most of the dogs were sensitised to multiple allergens other than *D. farinae*, and a good or bad response could have been influenced by other allergens in the vaccine. However, future studies are now warranted that compare the *D. farinae* specific IgG response to clinical outcome in a group of dogs that are mono-sensitised to that allergen.

Chapter 6

Development of quantitative real-time PCR assays to assess cytokine gene transcript expression in canine atopic dermatitis

6.1 Introduction

Recent interest has focused on the role of $CD4^+$ Th1 and Th2 cells in human atopic dermatitis (Grewe and others 1998; Leung 2000). Several studies have detected Th2 polarisation in the skin and PBMCs of atopic humans (van Reijsen and others 1992; Leung 1995; Neumann and others 1996; Kimura and others 1998a; Kimura and others 1998b) and mouse models (Spergel and others 1999; Vestergaard and others 1999). Although Th1 cytokines inhibit Th2 differentiation (Mosmann and Coffman 1989), the Th1-type cytokine IFN-γ, was found to be highly expressed in eczematous skin of the vast majority (80%) of human AD patients (Grewe and others 1994), suggesting that Th1 cells also participate in the pathogenesis of chronic atopic dermatitis. Furthermore, healthy individuals do not develop Th1 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 TGF-β (Muraille and Leo 1998; Koulis and Robinson 2000).

Imbalances in lymphocyte populations and cytokine production play an important role in the pathogenesis of human atopic dermatitis. Atopic lesions are dynamic and sequential activation of the Th2-cell subset during the initiation phase, followed by the Th1-cell subset has been hypothesized to account for the persistence of the inflammatory response (Grewe and others 1998). A biphasic pattern of cytokine expression has also been demonstrated after epi-cutaneous allergen challenge or atopy patch testing (APT) in humans (Grewe and others 1995; Thepen and others 1996). Using immunohistochemistry, biopsy sections from APT tested atopic patients were double stained for IL-4 and IFN-γ together with different membrane markers, and a clear dichotomy of the eczematous response to allergen in skin was

observed (Thepen and others 1996): in the initial phase IL-4 production by Th2 and Th0 cells was predominant over IFN-γ production by Th1 and Th0 cells, and in the late and chronic phases the situation was reversed and IFN-γ production predominated over IL-4 production (Thepen and others 1996).

To date, there are only a few studies looking at the cytokine profile in dogs with AD. A study using conventional RT-PCR investigating mRNA transcript expression of type-1 cytokines (IFN-γ, IL-2, IL-12p35, IL-12p40 and TNF-α) and type-2 cytokines (IL-4, IL-5, IL-6 and IL-10) in lesional and non-lesional skin specimens of atopic dogs and control skin samples from healthy dogs demonstrated a Th2-type cytokine pattern in one-fourth of atopic and a Th1-type pattern in one-fourth of healthy canine skin samples (Olivry and others 1999). Type-2 cytokines IL-4 and IL-5 were detected in 57.1% of lesional specimens, 40% of non-lesional samples, and in 25% of normal control specimens, whereas IL-6 and IL-10 transcripts were either rarely or not detected in atopic and control samples (Olivry and others 1999). Expression of type-1 cytokine IL-2 mRNA was less frequently detected in non-lesional atopic samples versus normal control specimens, and IL-12p40 transcripts were detected significantly more often in normal control than lesional atopic skin specimens (Olivry and others 1999).

Studies using semi-quantitative RT-PCR looking at the expression of Th2 cytokines (IL-4, IL-6), Th1 cytokines (IFN-γ, IL-2 and TNF-α) and immunosuppressive cytokines (TGF-β and IL-10) indicated an over-expression of IL-4 and under-expression of TGF-β in the skin of atopic dogs (Nuttall and others 2002a; Nuttall and others 2002b). Significantly higher levels of IL-4 mRNA were detected in both lesional and non-lesional atopic skin compared with healthy skin, whereas levels of TGF-β mRNA were significantly higher in healthy compared with atopic skin (Nuttall and others 2002a). A similar pattern of transcription for the type-1 cytokines IFN-γ, IL-2 and TNF-α was found and significantly higher levels of mRNA for each cytokine were detected in lesional atopic skin compared with either non-lesional atopic or healthy skin (Nuttall and others 2002a). Levels of IL-12p35 and IL-12p40 mRNA were highly variable in both atopic and healthy skin, and no difference was

found between the three groups of samples (Nuttall and others 2002a; Nuttall and others 2002b).

Another study using semi-quantitative RT-PCR examining IFN-γ, IL-4, IL-5 and IL-10 in the PBMCs of atopic and healthy dogs also demonstrated a Th2 cytokine pattern (Hayashiya and others 2002). In this study, The average IL-5 mRNA expression in atopic dogs was significantly higher than in control group, and levels of IL-4 mRNA tended to be higher in the atopic dogs as well (Hayashiya and others 2002). The IFN-γ mRNA expression level in atopic dogs was significantly lower than in control dogs, but expression of IL-10 did not differ between the groups (Hayashiya and others 2002).

A further study researching cytokine profiles of PBMCs from dogs experimentally sensitised to Japanese cedar pollen showed a Th2 polarisation and PBMC proliferation in response to the sensitising antigen (Fujiwara and others 2003). The expression level of cytokines IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IFN-γ, TGF-β and TNF-α were quantified using a real-time RT-PCR system, and significantly increased levels of TNF-α and IL-8 were detected in the experimentally sensitised group compared to the control group in the primary PBMCs, but IFN-γ was found to be decreased in the experimental group (Fujiwara and others 2003). The cytokine profile changed after PBMCs were stimulated with the crude Japanese cedar pollen antigen; the levels of IL-6 and TNF-α were expressed at greater quantities in the control group compared to the experimentally sensitised group, and the level of IL-2 mRNA in the PBMCs from the experimentally sensitised dogs was significantly higher than that in the PBMCs from the control dogs (Fujiwara and others 2003). The amount of IL-4 mRNA was below the lower detection limit in all of the sensitised and control dogs in primary PBMCs, but the amount of IL-4 mRNA was found to be markedly increased after PBMC stimulation with the offending antigen (Fujiwara and others 2003).

The most recent study investigated a panel of fourteen Th1, Th2, pro-inflammatory cytokines and chemokines in APT-tested high-IgE beagles experimentally sensitised

to house dust mites (Marsella and others 2006). The cytokine profiles analysed by real-time RT-PCR were studied before and after 6, 24, 48 and 96 hours of atopy patch testing. It was shown that IL-6 plays a role in early reactions followed by an increase of IL-13 and thymus and activation regulated chemokine (TARC), while IL-18 progressively increases in later reactions (Marsella and others 2006). The results support the hypothesis that, similar to human AD, there is varying skin expression of cytokine genes depending on lesion age.

Taken together, these studies suggest that a Th2 type cytokine profile may be apparent in canine atopic dermatitis, but the expression of Th2-type and Th1-type cytokines in AD is not mutually exclusive, suggesting that both Th-cell subsets contribute to the pathogenesis of canine atopic dermatitis. It can be argued that in both dogs and man, Th1- and Th2-type cytokines contribute to different stages of the development of skin lesions in atopic dermatitis patients. In such a model, a key element is the sequential activation of the Th2-cell subsets during the initiation phase, followed by the Th1-cell subset to account for the persistence of the inflammatory responses (Grewe and others 1994).

In addition to T-cell derived cytokines, other mediators may be involved in inflammatory skin diseases. Inducible nitric oxide synthase (iNOS) is involved in the generation of nitric oxide (NO), a dynamic molecule utilised through the animal kingdom as a signalling or toxic agent between cells. NO exhibits complex functions, and is also involved in innate immunity as a toxic agent towards infectious organisms and can induce or regulate death and function of host immune cells, thereby regulating specific immunity (Coleman 2001). Expression of iNOS mRNA was found to be decreased in AD, as compared with psoriasis (Nomura and others 2003), but the immunoreactivity of iNOS was significantly more extensive in AD skin than in contact dermatitis skin (Rowe and others 1997). However, the role of iNOS in the context of AD is far from fully understood.

Many cellular decisions concerning survival, growth and differentiation are reflected in altered patterns of gene expression and the ability to quantify transcription levels

of specific genes has always been central to research into gene function and its role in disease pathogenesis (Zamorano and others 1996). The fluorescence-based kinetic real-time RT-PCR procedure significantly simplifies the process of producing reproducible quantification of mRNA, and is an excellent tool in investigating the expression of various genes actively involved in the immunopathogenesis of atopic dermatitis. Although it would have been ideal to include all potential immune response genes in this study, it was not practical due to logistical, funding and time limitations. Therefore, one representative gene from each subset of the immune system was chosen for real-time RT-PCR. Based on the findings of previous studies, cytokines shown to differ between atopic and control groups were selected as key examples, and they were the Th1-type cytokine IFN-γ, the Th2-type cytokine IL-4, and the immunosuppressive regulatory cytokine TGF-β. iNOS was also included as a representative of the innate immune response.

The aim of this study was to further explore, using new methodologies, the possibility that canine atopic dermatitis is associated with polarised Th1/Th2 responses as seen in humans, and to investigate the role of iNOS in AD.

6.2 Development, validation and optimisation of methodology

The purpose of this section was to develop, validate and optimise quantitative real-time reverse transcription mediated polymerase chain reaction (qRT-PCR) to detect mRNA for the four cytokines mentioned in 6.1, including interleukin-4 (IL-4), interferon-γ (IFN-γ), transforming growth factor-β (TGF-β), inducible nitric oxide synthase (iNOS) as well as the housekeeping gene 18S ribosomal RNA (rRNA). Conventional PCR was used to amplify the target gene first. The PCR product was then cloned and DNA sequencing was used to confirm its identification. The cloned cytokine fragment was then used to generate a standard curve in real-time PCR experiments in order to calculate the absolute copy number of the cytokine transcript being examined.

6.2.1 Canine cytokine amplification using positive control samples

The primer sequences used to amplify target canine cytokine transcripts were designed using an online primer design program Primer3 (Chapter 2.8.1). For real-time PCR, primer pairs were designed with optimal amplicon size of 100 bp. A pair of outer primer flanking target sequences for real-time PCR was also obtained for each cytokine. The amplification products of the outer primers were used for cloning. Positive template DNA samples expressing IL-4, IFN-γ and TGF-β were selected using primers previously designed by Nuttall (Nuttall and others 2002a; Nuttall and others 2002b). Screening of iNOS template positive samples was done using newly designed outer primers. The published primer pairs specific for the housekeeping gene 18S rRNA was used to detect template containing samples.

RNA extracted from the popliteal lymph nodes of healthy dogs was reverse transcribed into complementary DNA using random hexamer primer (Chapter 2.7.5). The lymph nodes are secondary lymphoid organs that function through adult life. These organs are rich in macrophages and dendritic cells that trap and process antigens, and in T and B lymphocytes, which mediate the immune responses. They were therefore likely to be a good source of the cytokines that were of interest.

Standard PCRs were performed in 50 μl volumes containing 2 μl cDNA, 400 nM forward and reverse primers, 2.5 units *Taq* DNA polymerase in a reaction buffer composed of 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.2 mM each dNTPs (Chapter 2.8.2). The reaction conditions consisted of initial denaturing at 94°C for 2 min, then cycles of denaturing at 94°C for 30 sec, primer annealing for 30 sec and elongation at 72°C for 30 sec, before a final elongation at 72°C for 10 min (Chapter 2.8.2). Results of standard PCRs were visualised in agarose gel containing 0.5 μg/ml ethidium bromide and recorded on the image station (Chapter 2.8.3).

6.2.2 Conventional PCR optimisation

Factors influencing the performance of a real-time PCR include divalent metal (magnesium ion, Mg^{2+}) concentration, primer concentration, dNTP concentration,

buffer pH and annealing temperature. Two of the most important ingredients influencing the results of a PCR reaction are the buffer and the magnesium concentration. A panel of 16 buffers (PCR optimisation kit, Roche) comprising different magnesium concentrations and pH was used to evaluate appropriate reaction conditions for each pair of primers.

16 reactions differing only in the composition of magnesium concentrations (ranging from 1.0 mM to 2.5 mM) and buffer pH (ranging from 8.3 to 9.2) (Table 6.1) were set up for each set of primers and the results are shown in Figure 6.1.

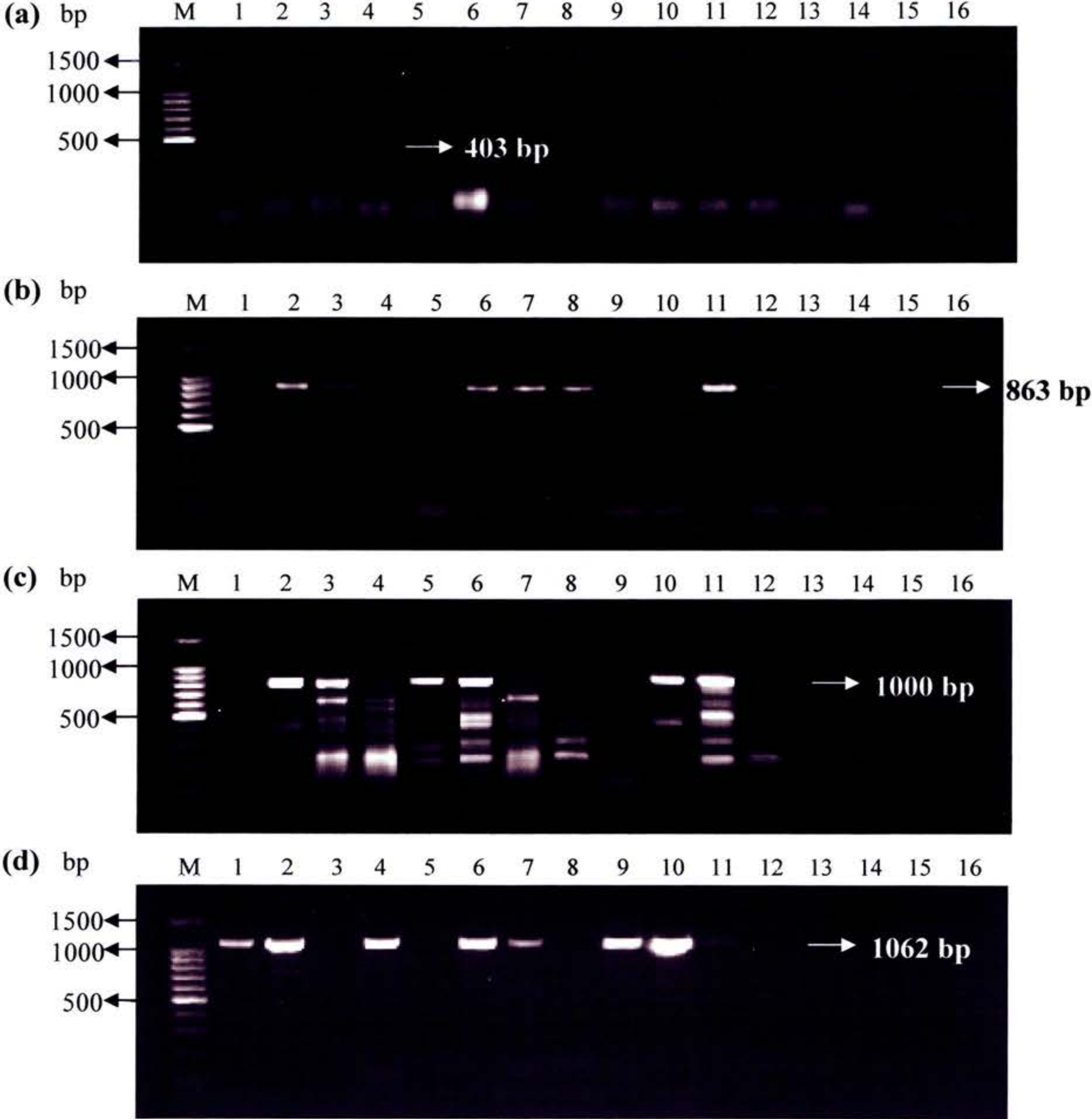
Reactions with expected amplicon size showing a band with strong intensity and reactions without non-specific amplification were chosen for each pair of primers. For IL-4 (Figure 6.1 a) only a weak band with MW of 400 bp in lane 4 was visible. A magnesium concentration of 2.5 mM with buffer pH of 8.3 was chosen for the amplification of canine IL-4. For IFN-γ (Figure 6.1 b), the reaction conditions in lane 11 comprising buffer with 2.0 mM Mg^{2+} and pH 8.9 yielding the strongest band intensity was selected. For TGF-β (Figure 6.1 c), designed primers amplified non-specifically under most buffer conditions. However, the reaction conditions in lane 10 were chosen because a band with the expected amplicon size was present and non-specific binding was minimal. Buffer with a magnesium concentration of 1.5 mM and pH of 8.9 was used for the amplification of canine TGF-β. Several reaction conditions yielded specific amplification of iNOS (Figure 6.1 d). The buffer comprising a magnesium concentration of 1.5 mM and pH of 8.9 yielded the strongest signal and was therefore selected.

Table 6.1 Concentrations of $MgCl_2$ and pH-value for buffers 1-16

pH	**$MgCl_2$ concentration (mM)**			
	1.0	**1.5**	**2.0**	**2.5**
8.3	1	2	3	4
8.6	5	6	7	8
8.9	9	10	11	12
9.2	13	14	15	16

Figure 6.1 PCR optimisation experiment evaluating reaction $MgCl_2$ concentration and buffer pH

16 buffers, each containing 100 mM Tris-HCl, 500 mM KCl, 1-2.5 mM $MgCl_2$. Detailed concentrations of $MgCl_2$ and pH-value for each buffer are listed in Table 6.1. Expected amplicon size is 403 bp for IL-4 (a); 863 bp for IFN-γ (b); 1000 bp for TGF-β (c); and 1062 bp for iNOS (d). M: molecular weight marker; bp: base pairs.



6.2.3 Cloning of canine cytokine gene fragments

Freshly amplified PCR products were cloned into the linear plasmid vector pCR®4-TOPO® and re-introduced into chemically competent *E. coli* (Chapter 2.6.6). The *E. coli* transformant cells harbouring target inserts were then plated onto LB agar plates and incubated overnight at 37°C in an incubation chamber. A single colony was then inoculated into 5 ml LB medium and incubated overnight at 37°C with vigorous shaking at 250 rpm. Purified plasmid DNA was digested with *Eco*R I to release the inserted fragment and assessed with gel electrophoresis. After confirming that the insertion had occurred, DNA sequencing was performed to confirm the identity of the inserted sequence.

DNA fragments of canine IL-4 (403 bp), iNOS (1062 bp), TGF-β (1000 bp) and IFN-γ (863 bp) were cloned and analysed (Figure 6.2). Plasmid DNA (3956 bp) and inserted fragments were separated and visualised on the gel. IFN-γ, TGF-β and iNOS appeared to be cloned into the plasmid whereas IL-4 fragment was not inserted in the vector (Figure 6.2). DNA sequencing of purified plasmid DNA with IFN-γ or iNOS insertions confirmed their identity, whereas the sequencing result of the TGF-β clone revealed a segment of the *E. coli* genome.

The cloning of the 403-bp canine IL-4 was not successful. The problem could have arisen from poor primer design even though known design criteria were met. In the IL-4 PCR optimisation experiment, a weakly visible band was detected when the reaction was performed in 2.5 mM $MgCl_2$ with buffer pH 8.3 (Figure 6.1a). It was obvious that the amplification efficiency was not ideal, therefore the resultant PCR product was not suitable for cloning. Gel-purification of the target PCR fragment was also performed prior to cloning, but possible dilution of the DNA was likely, making successful cloning even more difficult to achieve. Therefore, another pair of primers targeting the canine IL-4 flanking 114-bp fragment was chosen for attempted cloning.

Cloning of the 1000-bp canine TGF-β was unsuccessful the first time it was attempted. The sequencing result revealed that the identity of the insert belonged to the *E. coli* genome. Chemically competent *E. coli* were used as transformants, but how and when contamination occurred remained unknown.

Limited sequence data of dog 18S rRNA was available, and only a set of primers designed to amplify canine 18S rRNA with an amplicon size of 96 bp has been published to date. Therefore, the same primer pair used in real-time RT-PCR was used for cloning.

A second attempt at cloning and sequencing was performed for IL-4, TGF-β and 18S rRNA. After the standard cloning procedure and bacterial cultural expansion, plasmid DNA was purified and analysed by *Eco*R I digestion and gel electrophoresis. A 1000-bp band was seen for TGF-β clones (Figure 6.3) whilst the 96-bp and 114-bp inserts for canine 18S rRNA and IL-4 were not visualised in the gel. It was possible that those small fragments had migrated out of the gel. Although the expected cloned fragments were not seen on the gel electrophoresis analysis, DNA sequencing of these clones was performed, and sequencing results confirmed the identity of inserted sequences to be canine TGF-β (1000 bp), 18S rRNA (96 bp) and IL-4 (114 bp) (Table 6.2).

Figure 6.2 Evaluation of canine IL-4, IFN-γ, TGF-β and iNOS clones

Purified plasmid DNA was analysed by *Eco*R I digestion and agarose gel electrophoresis. 4 randomly selected single colonies expanded in LB broth overnight were chosen for each gene clone. The 3956-bp plasmid vector as well as the insert of interest were seen in IFN-γ (b), TGF-β (c) and iNOS (d) clones, whereas insertion of the IL-4 (a) gene fragment did not occur. M: molecular weight marker; kb: kilo bases; bp: base pairs.

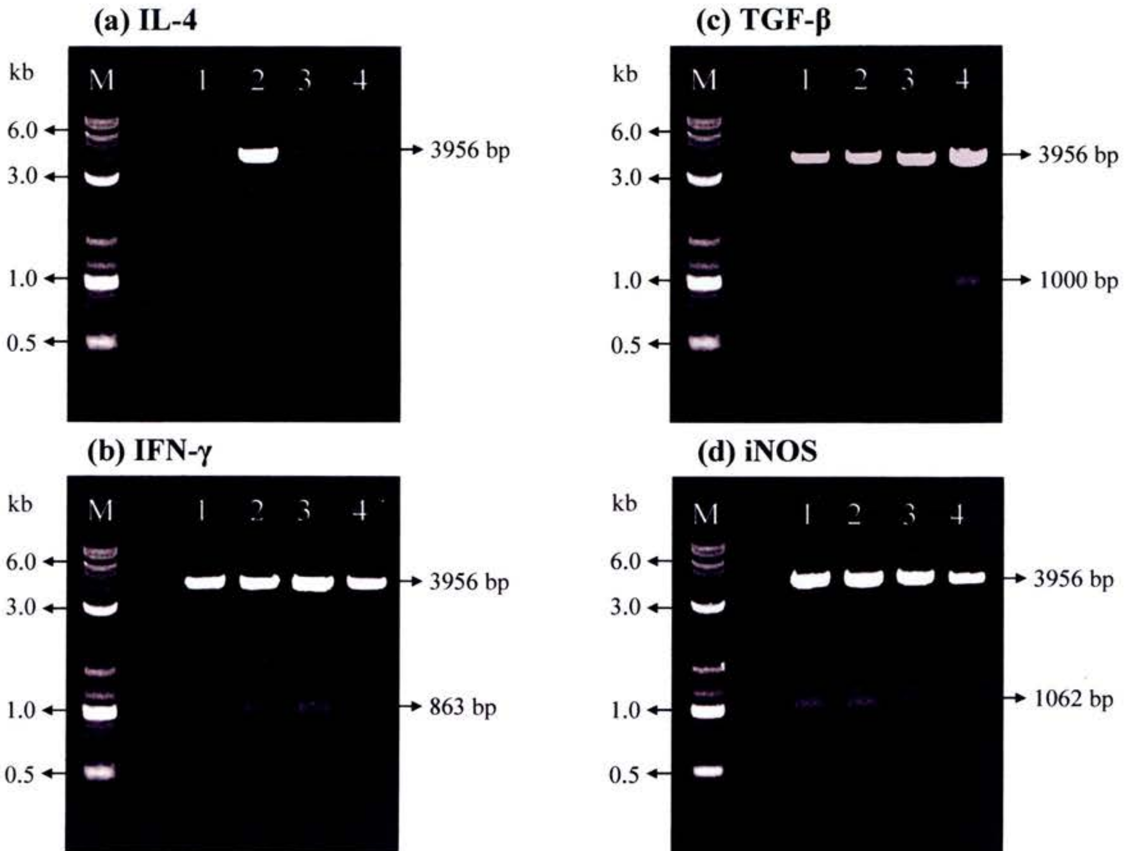


Figure 6.3 Evaluation of canine TGF-β, 18S rRNA and IL-4 clones

Purified plasmid DNA was analysed by *Eco*R I digestion and agarose gel electrophoresis. 4 randomly selected single colonies expanded in LB broth overnight were chosen for each gene clone. The 3956-bp vector was present in every well, whilst excessive amount of plasmid DNA was loaded in lanes 1-3. The expected target insert of 1000-bp for TGF-β was seen in lanes 1-3 and absent in lane 4. The 96-bp and 114-bp inserts of 18S rRNA and IL-4 could not be visualised in the gel. M: molecular weight marker; kb: kilo bases; bp: base pairs.

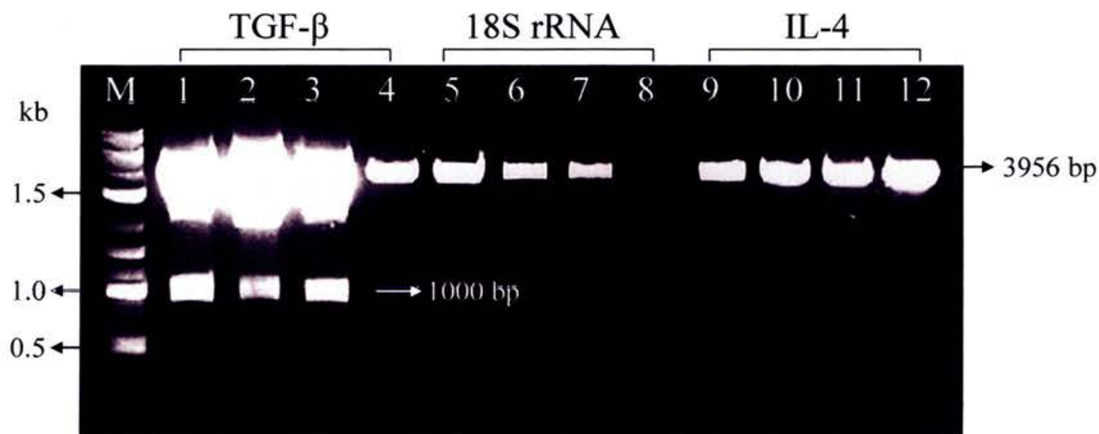


Table 6.2 BLAST result confirming cloning sequence

Gene	Cloned size (bp)	Length of sequence obtained (bp)	Score[1] (Bits)	E value[2]	Identities	Gaps	Sequence producing best alignment	
							Accession number	Gene identity
IFN-γ	863	577	1112	0.00	575/577 (99%)	2/577 (0%)	NM_001003174	Canis familiaris interferon, gamma (IFNγ), mRNA
iNOS	1062	650	1217	0.00	644/650 (99%)	3/650 (0%)	AF032909	Canis familiaris inducible nitric oxide synthase mRNA, partial cds[3]
TGF-β	1000	104	190	8e-46	103/104 (99%)	1/104 (0%)	AF349538	Canis familiaris transforming growth factor beta 1 mRNA, partial cds.
IL-4	114	114	226	6e-57	114/114 (100%)	0/114 (0%)	NM_001003159	Canis familiaris interleukin 4 (IL4), mRNA
18S rRNA	96	96	190	1e-45	96/96 (100%)	0/96 (0%)	AY262732	Canis familiaris 18S ribosomal RNA gene, partial sequence

[1] The bit score gives an indication of how good the alignment is; the higher the score, the better the alignment. In general terms, this score is calculated from a formula that takes into account the alignment of similar or identical residues, as well as any gaps introduced to align the sequences.

[2] The Expect value (E) is a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size. It decreases exponentially with the Score (S) that is assigned to a match between two sequences. Essentially, the E value describes the random background noise that exists for matches between sequences.

[3] cds: coding sequence

6.2.4 Optimisation of PCR annealing temperature

Factors influencing the performance of a real-time PCR include divalent metal (i.e. Mg^{2+}) concentration, primer concentration, dNTP concentration and annealing temperature. Non-specific amplifications can be reduced by increasing annealing temperature; therefore, annealing temperature was first optimised for each canine gene transcript of interest. The optimised temperature of each PCR reaction was determined using the temperature gradient function of the thermal cycler (GeneAmp® PCR system 2400; Perkin-Elmer, Cambridge, UK). Eight reaction tubes, using the same dilution of cloned DNA fragment as template, were run at a range of annealing temperatures between 55 and 65°C. The highest annealing temperature with successful PCR amplification is chosen for qRT-PCR because of reduced mis-priming events and non-specific amplifications. The results indicated that 65°C of annealing temperature was suitable for all cytokines in real-time amplification (Figure 6.4).

Figure 6.4 Annealing temperature optimisation of real-time PCR primers

Eight reactions were run at different annealing temperature ranging from 55°C (lane 1) to 65°C (lane 8) for the primers for canine IL-4 (a), IFN-γ (b), TGF-β (c), iNOS (d) and 18S rRNA. The annealing temperature is 56.4°C, 57.8°C, 59.3°C, 60.7°C, 62.1°C and 63.6°C in lanes 2 to 7. Amplification results were analysed by agarose gel electrophoresis and visualised in UV light. The results indicated that 65°C annealing temperature was suitable for all cytokines in real-time amplification.

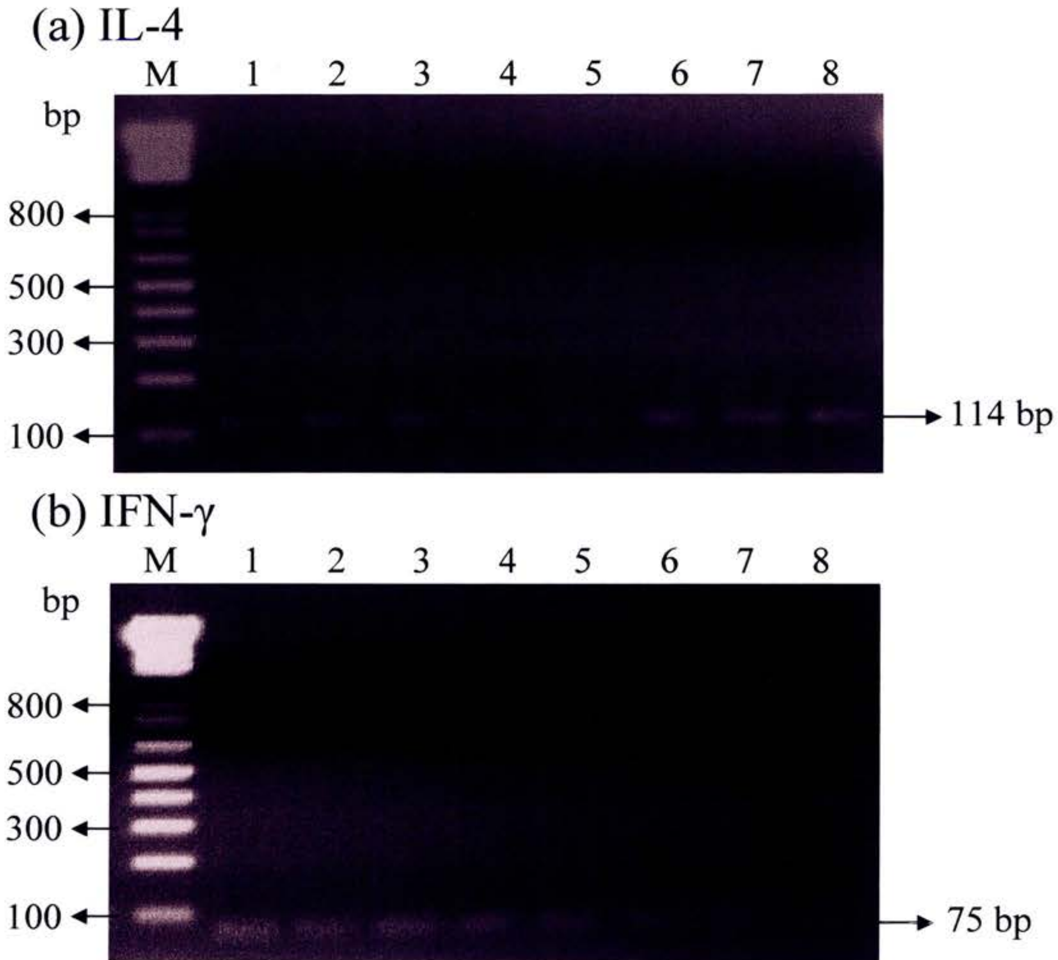
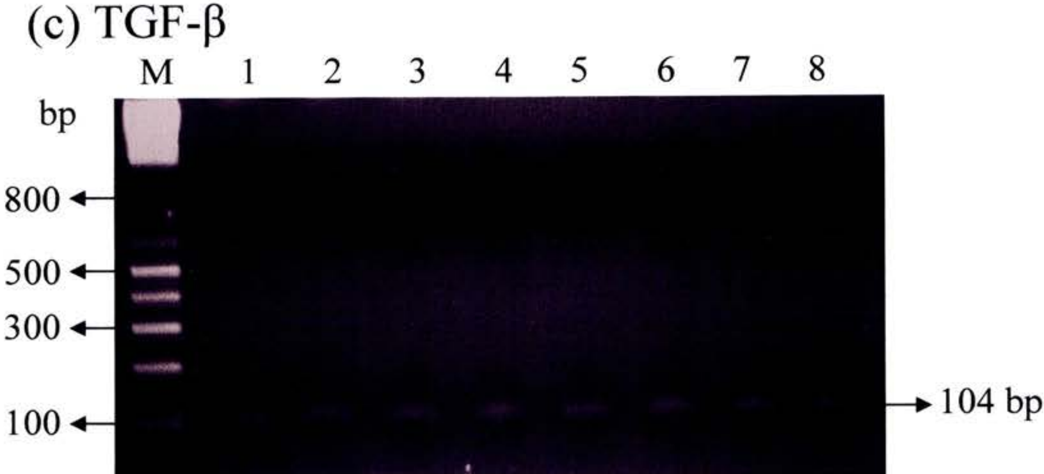
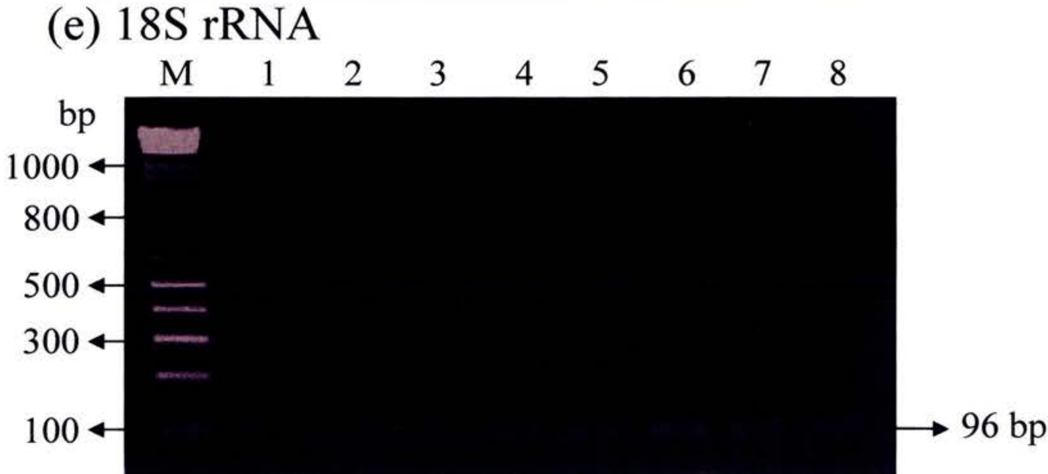


Figure 6.4 contd. Annealing temperature optimisation of real-time PCR primers





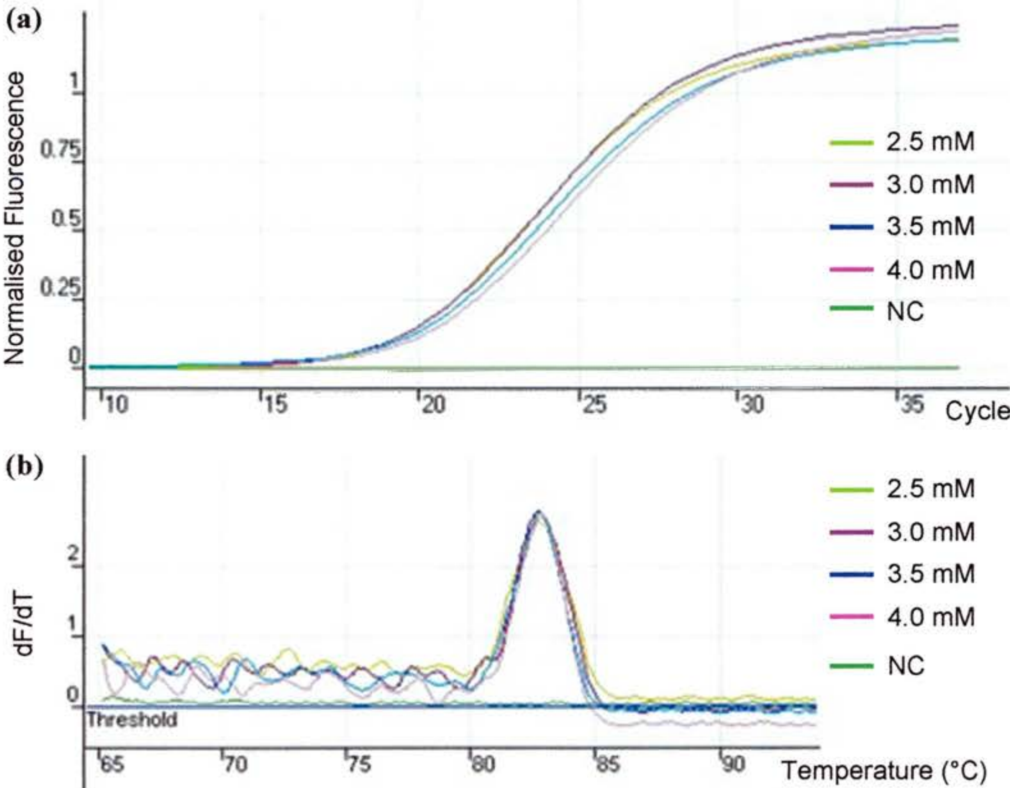
6.2.5 Optimisation of magnesium chloride concentration

Mg^{2+} and dNTP concentrations require strict control, because Mg^{2+} affects enzyme activity, and imbalanced dNTP mixtures will reduce polymerase fidelity (Eckert and Kunkel 1991). In addition, Mg^{2+} increases the melting temperature (Tm) of double-stranded DNA, and forms soluble complexes with dNTPs to produce the actual substrate that the polymerase recognises. Therefore, high concentration of dNTPs interferes with polymerase activity and affects primer annealing by reducing free Mg^{2+}.

4 duplicate reactions differing only in the composition of magnesium concentrations (ranging from 2.5 mM to 4.0 mM) were set up for each set of primers. 10^5 copies of the cloned template for each cytokine were used as template for real-time optimisation experiments. The optimal Mg^{2+} concentration was 3.0 mM for 18S rRNA, TGF-β and IL-4, and 4.0 mM for IFN-γ and iNOS. The representative results of 18S rRNA optimisation are shown in Figure 6.5.

Figure 6.5 Magnesium chloride concentration optimisation for 18S rRNA using SYBR Green dye and a melting curve analysis of the resulting products

(a) A representative experiment in which the total fluorescence was measured at the end of each cycle. The negative control contains no input cDNA. All samples were amplified simultaneously for 45 cycles and the fluorescence was measured at the end of each extension step. (b) A melting curve analysis in which melting peaks were determined by plotting the negative derivative of fluorescence emitted by each sample during a melting curve analysis in which PCR products were slowly melted and fluorescence was determined continuously. The product of interest of 18S rRNA had a Tm of 82.8°C. dF: change in fluorescence; dT: change in temperature; NC: negative control.



6.2.6 Generation of real-time RT-PCR standard curves

The standard curve for each individual amplicon was constructed from purified plasmid dsDNA of known concentration. This curve was then used as a reference standard for extrapolating quantitative information for mRNA targets of unknown concentrations. Ideally RNA standards should be used for absolute quantification of mRNA; however, *in vitro* transcribed RNA standards are difficult to accurately quantify and their stability could be a source of variability in the final analysis. Therefore, plasmid DNAs were used to construct a standard curve for quantification. The variation introduced due to variable RNA inputs was corrected by normalisation to a housekeeping gene 18S rRNA.

The concentration of the plasmid was measured using a spectrophotometer, converted to g/µl by multiplying a factor of 10^{-9}, and the corresponding copy number was calculated using the following equation:

$$\textbf{copy number (copies/}\mu\textbf{l)} = \frac{\textbf{6.02} \times \textbf{10}^{23} \textbf{ (copies/mol)} \times \textbf{DNA concentration (g/}\mu\textbf{l)}}{\textbf{molecular weight of plasmid DNA (g/mol)}}$$

where molecular weight = (insert bp + vector 3956 bp) × 660 (dalton/bp)

The concentrated DNA was then diluted over several orders of magnitude by accurate pipetting. The stability of the diluted standards was important, so diluted standards were divided into small aliquots, stored at -70°C, and thawed only once before use. A hundred-fold dilution series of cloned cytokine fragments was produced in duplicate for each cytokine assay. The reaction efficiencies were calculated from the slope of the threshold cycle vs. copy number graphs and were 96.99 %, 95.14 %, 96.32 %, 93.58 % and 97.07 % with correlation coefficients of 0.9981, 0.9995, 0.9982, 0.9996 and 0.9901 for 18S rRNA (Figure 6.6), TGF-β (Figure 6.7), IL-4 (Figure 6.8), IFN-γ (Figure 6.9) and iNOS (Figure 6.10) respectively.

6.2.7 Melting curve

The intercalating dye SYBR Green used in real-time PCR fluoresces intensely when associated with double stranded DNA. However, any double stranded product, including primer-dimers, is detected and false positives could occur. A melting curve was used to identify formation of primer-dimer and non-specific signals.

A melting curve was produced by heating the samples from 65°C to 95°C in 0.3°C increments with a dwell time at each temperature of 10 seconds during which the fluorescence data were collected. The melting temperatures of the product was determined with the Rotor-Gene Software (version 6: Corbett Research) using a rate of change of fluorescence (dF/dT) vs. temperature graph (Figures 6.6-6.10 b).

Figure 6.6 Amplification of canine 18S rRNA gene in 100-fold dilutions of template

(a) Amplification plot showing the change in fluorescence of SYBR® Green I dye plotted versus cycle number. (b) Melt curve analysis of online PCR signals. The 18S rRNA amplicon had a Tm of 82-83°C. (c) Standard curve was generated. Reaction efficiency and correlation coefficient (R^2) values were noted. NC: negative control; Norm. Fluoro.: normalised fluorescence; dF: change in fluorescence; dT: change in temperature; deg.: degree in °C.

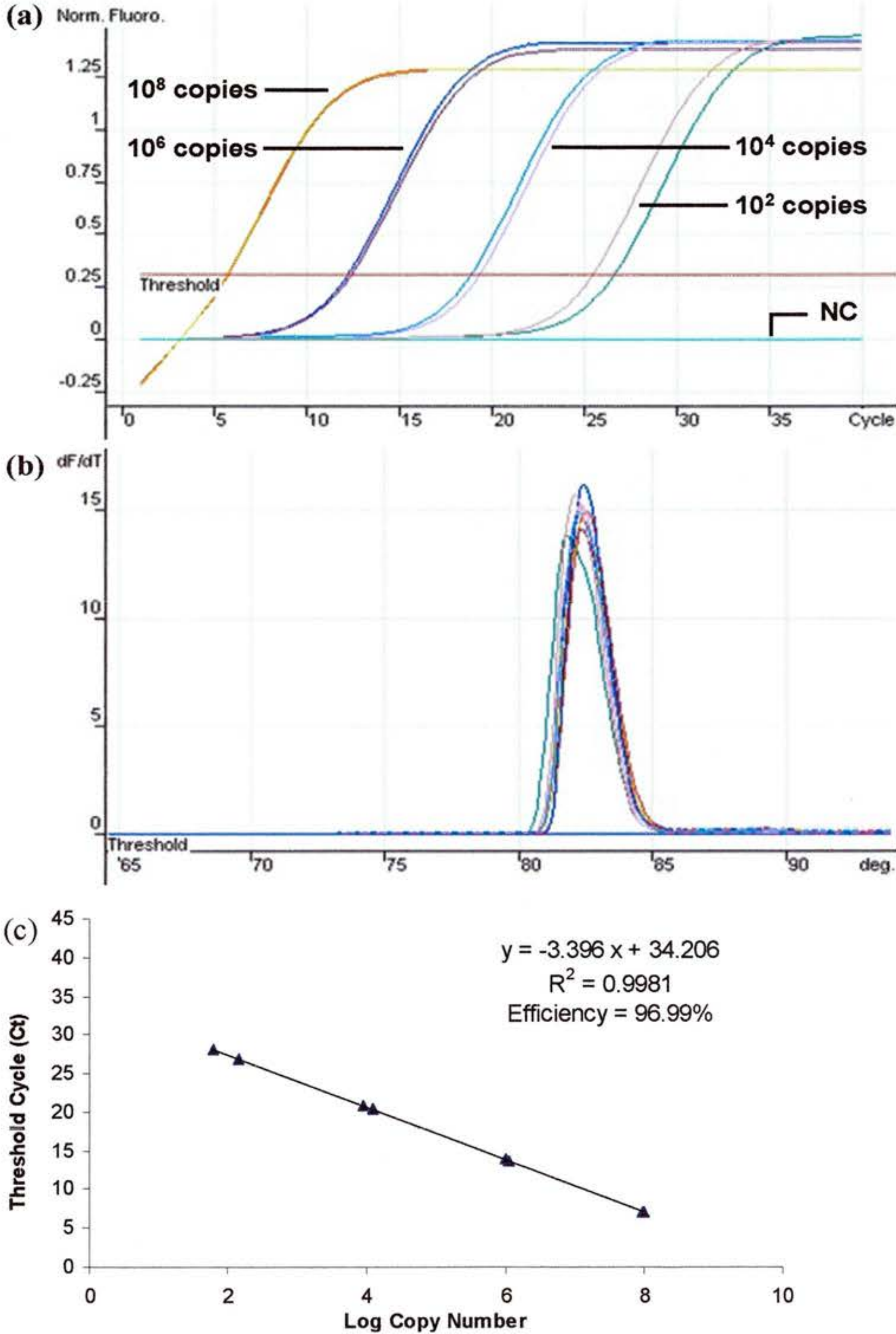


Figure 6.7 Amplification of canine TGF-β gene in 100-fold dilutions of template

(a) Amplification plot showing the change in fluorescence of SYBR® Green I dye plotted versus cycle number. (b) Melt curve analysis of online PCR signals. The TGF-β amplicon had a Tm of 85-86°C. Non-specific signals were detected in reactions with 10^4 and 10^2 copies of template. (c) Standard curve was generated. Reaction efficiency and correlation coefficient (R^2) values were noted. NC: negative control; Norm. Fluoro.: normalised fluorescence; dF: change in fluorescence; dT: change in temperature; deg.: degree in °C.

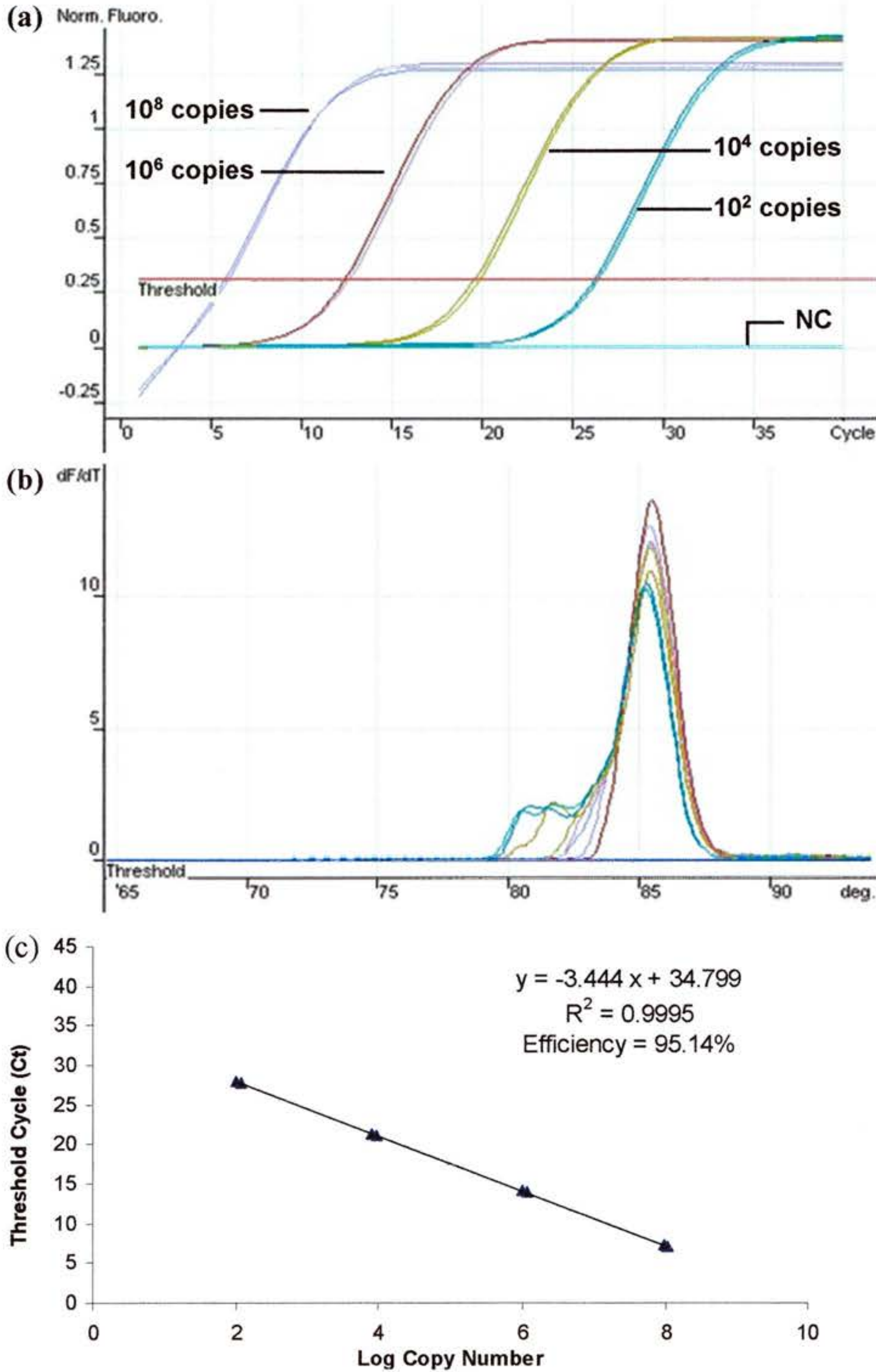


Figure 6.8 Amplification of canine IL-4 gene in 100-fold dilutions of template

(a) Amplification plot showing the change in fluorescence of SYBR® Green I dye plotted versus cycle number. (b) Melt curve analysis of online PCR signals. The IL-4 amplicon had a Tm of 79-80°C. (c) Standard curve was generated. Reaction efficiency and correlation coefficient (R^2) values were noted. NC: negative control; Norm. Fluoro.: normalised fluorescence; dF: change in fluorescence; dT: change in temperature; deg.: degree in °C.

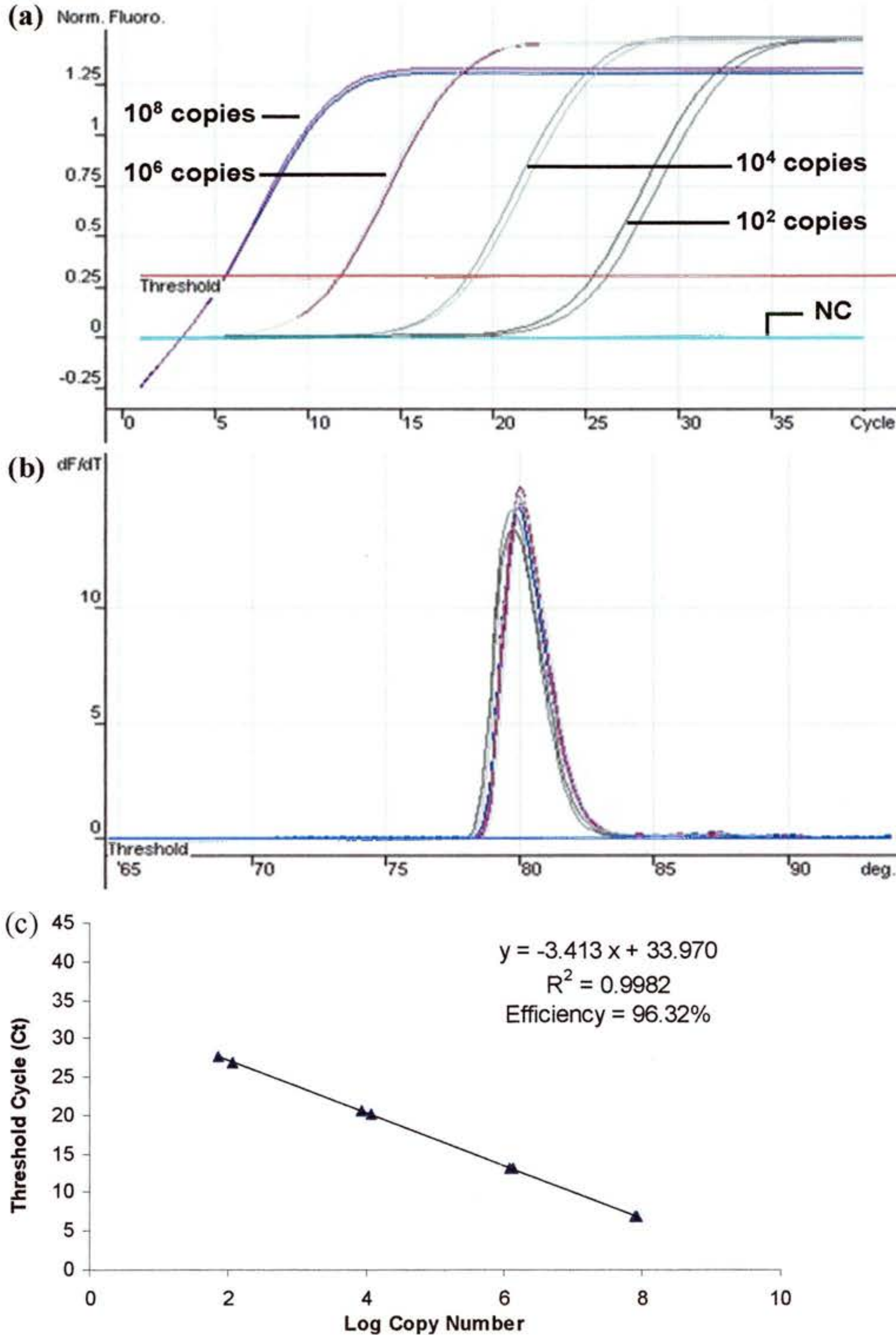


Figure 6.9 Amplification of canine IFN-γ gene in 100-fold dilutions of template

(a) Amplification plot showing the change in fluorescence of SYBR® Green I dye plotted versus cycle number. (b) Melt curve analysis of online PCR signals. The IFN-γ amplicon had a Tm of 81-82°C. (c) Standard curve was generated. Reaction efficiency and correlation coefficient (R^2) values were noted. NC: negative control; Norm. Fluoro.: normalised fluorescence; dF: change in fluorescence; dT: change in temperature; deg.: degree in °C.

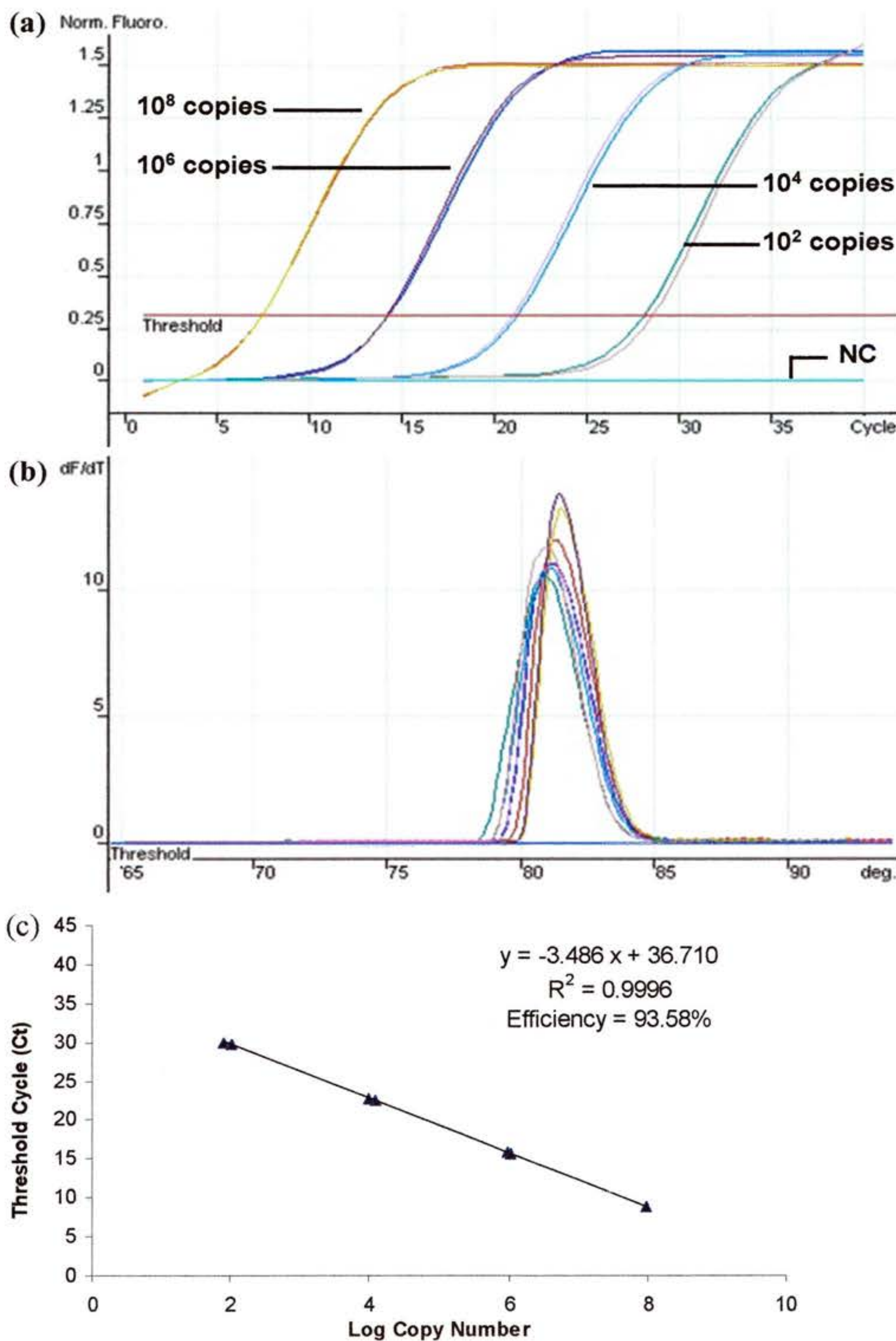
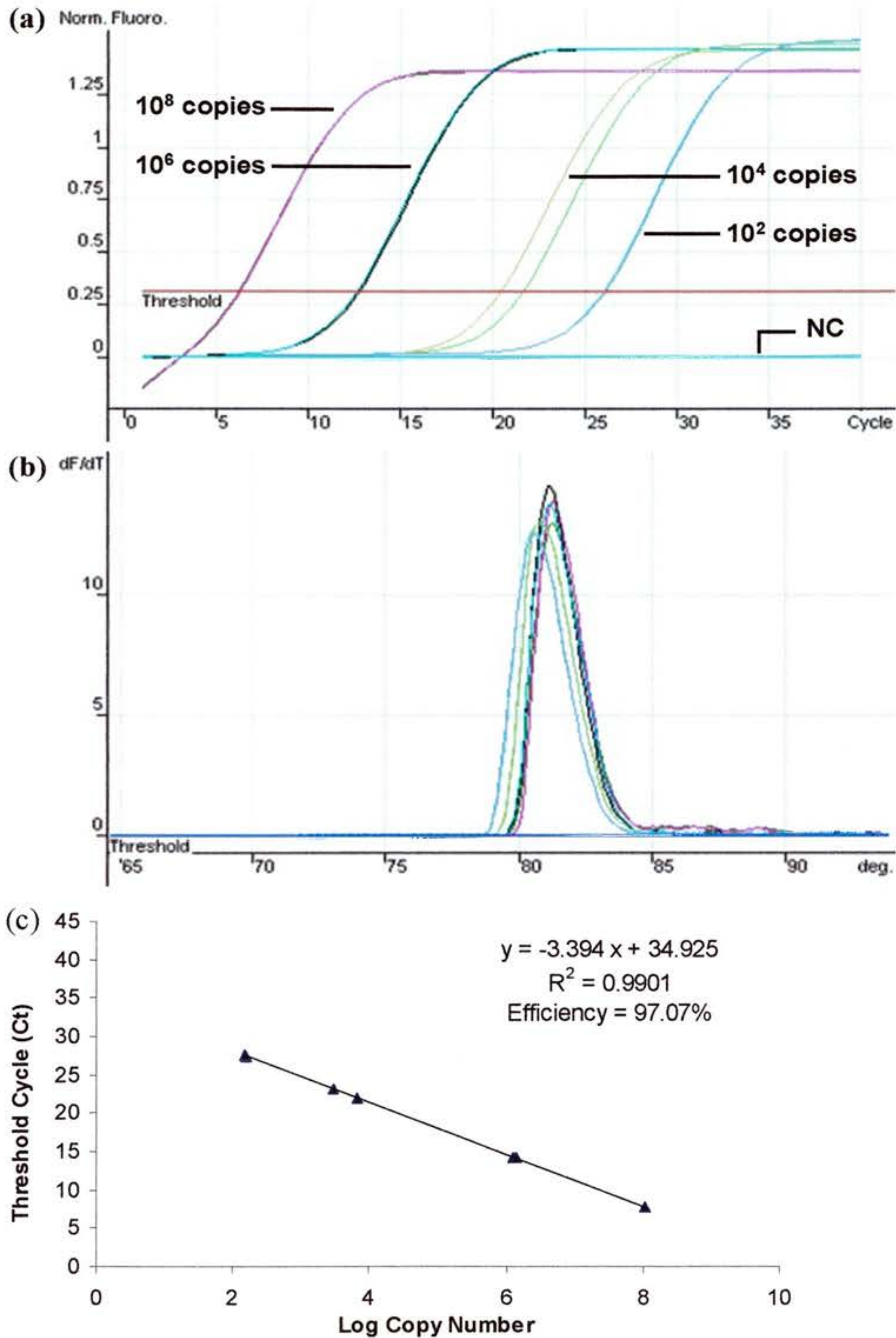


Figure 6.10 Amplification of canine iNOS gene in 100-fold dilutions of template

(a) Amplification plot showing the change in fluorescence of SYBR® Green I dye plotted versus cycle number. (b) Melt curve analysis of online PCR signals. The iNOS amplicon had a Tm of 81-82°C. (c) Standard curve was generated. Reaction efficiency and correlation coefficient (R^2) values were noted. NC: negative control; Norm. Fluoro.: normalised fluorescence; dF: change in fluorescence; dT: change in temperature; deg.: degree in °C.



6.2.8 Cytokine copy number calculation

When assaying the canine skin samples for cytokine expression, the corresponding standard series was run under the same conditions. The absolute copy numbers for the cytokine in question and the housekeeping gene (18S rRNA) was determined automatically by the real time machine software by inputting the Ct value of the sample into the standard curve. Comparing samples requires normalisation to compensate for difference in the amount of biological material in the tested samples. rRNAs, which constitute 85-90% of total cellular RNA, are useful internal controls, as the various rRNA transcripts are generated by a distinct polymerase and their levels are less likely to vary under conditions that affect the expression of mRNAs (Barbu and Dautry 1989). It has also been shown that rRNAs are more reliable than other commonly used housekeeping genes, i.e. β-actin and GAPDH in human and murine cells (de Leeuw and others 1989; Mansur and others 1993; Bhatia and others 1994; Zhong and Simons 1999). 18S rRNA was therefore selected as the internal control.

The variation introduced due to variable RNA inputs was corrected by normalisation to 18S rRNA using the following equation:

Adjusted absolute copy number

= target copy number / 18S rRNA copy number * 1,000,000

6.3 Results

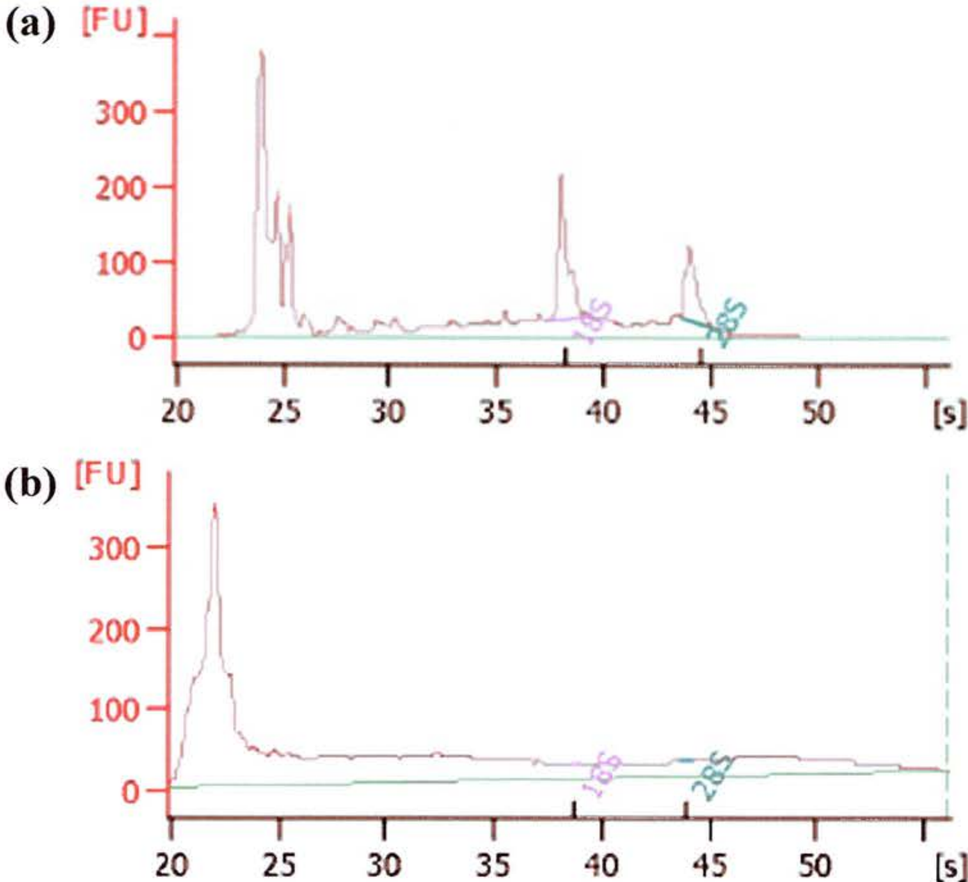
6.3.1 Archive sample RNA quality assessment

RNAs extracted from skin samples from atopic and healthy dogs were collected by Dr. Nuttall during 2000-2001 and had been stored at -70°C (Chapter 2.2.1). The Agilent 2100 Bioanalyzer was used to assess the quality of archived sample RNAs (Chapter 2.7.4). In this assay, RNA is combined with a sample buffer containing a fluorescent dye that intercalates into the RNA and is excited by an internal 635 nm diode laser. Data output is in the form of an electropherogram, which graphically depicts spikes in fluorescence over time; the larger the peak, the more intact ribosomal subunits are in the sample. If a sample is degraded, subunits will show a smaller degree of fluorescence that is spread out over a longer period of time, indicating a greater variety of sizes in the sample.

Two representative results of RNA quality assessment illustrated in electropherograms are shown in Figure 6.11. The big peak seen at 22-24 seconds is the marker. The peaks appearing at 24-29 seconds could be 5S RNA and transfer RNA. The trizol RNA extraction method used does not remove 5S and tRNA in contrast to many column-based RNA extraction kits that do remove these small RNA species. Distinct peaks for 18S and 28S rRNA were detected in 25 of the 35 samples and degradation was more evident in 10 samples. However, the archived RNA samples were not of the highest quality and some degradation had occurred because rRNA peaks did not dominate the fluorescence of these samples and various other peaks were also present. Several reasons for unexpected peaks other than RNA degradation include contamination of dust in the working environment, contamination of particles from the cell during RNA extraction, bubbles forming during pipetting or during heating of the gel through the electrical current and inappropriate gel filtration. These results indicated that the archived RNA samples used for the real-time RT-PCR assays were partially degraded and interpretation of real-time results would therefore require caution.

Figure 6.11 RNA quality assessment measured by the Agilent 2100 Bioanalyzer

Representative electropherograms showing good quality (a) and poor quality (b) RNA. Clear peaks for 18S and 28S rRNA were seen in most RNA samples. In the lower part of the graph, 5S RNA and transfer RNA can also be seen. Various unexpected bands were present in most samples, indicating possible RNA degradation. FU: fluorescence; S: time in seconds.



6.3.2 Cytokine transcript expression in healthy and atopic dogs

Graphical illustrations of real-time fluorescent RT-PCR reactions are shown in Figure 6.12 for 18S rRNA, Figure 6.13 for TGF-β, Figure 6.14 for IL-4, Figure 6.15 for IFN-γ and Figure 6.16 for iNOS. The absolute copy numbers of cytokine transcripts were automatically derived by the real-time PCR software from the standard curves, consisting of known copy numbers of plasmid DNA as calculated using the equation in Chapter 6.2.6. The raw data obtained from the real time machine are shown in the appendix at the back of this thesis. Levels of cytokine mRNA were quantified in most of the samples collected in this study (Table 6.3). Biopsies from all dogs were found to express 18S rRNA, TGF-β, IL-4, IFN-γ and iNOS. The most abundant transcripts were those encoding 18S rRNA and iNOS, and the least abundant transcripts were those encoding IL-4, TGF-β and IFN-γ.

Descriptive statistics of the normalised absolute copy numbers are summarised in Table 6.4, and illustrated in Figure 6.17. Overall, the results tended to show trends rather than statistically significant differences, and there was marked variation in copy numbers between samples. For TGF- β, copy numbers tended to be higher in healthy and non-lesional atopic skin compared to lesional skin, but the difference did not reach statistical significance (p=0.1052 and 0.0664 respectively). Expression of IL-4 was generally low in all three groups, although when it was present, higher levels were observed in atopic skin. For IFN-γ, copy numbers were higher in atopic skin compared to the controls and a significant difference was seen between lesional and healthy skin (p=0.0338); expression of IFN-γ was also higher in non-lesional atopic skin compared to healthy controls (p=0.0710). With iNOS, copy numbers were highest in healthy and non-lesional skin but appeared to be lower in lesional atopic skin.

Figure 6.12 Detection and quantification of 18S rRNA by real-time RT-PCR

PCR cycle numbers were plotted against normalised fluorescence intensities on a logarithmic scale, for better illustration of the exponential phase of the PCR (a). Slopes of the exponential amplification phases are highly reproducible and independent of template concentration. The horizontal red line indicates the fluorescence threshold of amplification detection at normalised fluorescence = 0.0802, set within the exponential phase and determined by real-time software. Absolute copy numbers of unknown samples (dots in red) were derived from data of standards (dots in blue) (b). The value of Ct ranges from 4.97 to 21.07 with a mean of 8.683. The copy number per µl ranges from 75,984,061 to 3,190 with a mean of 17,201,548.

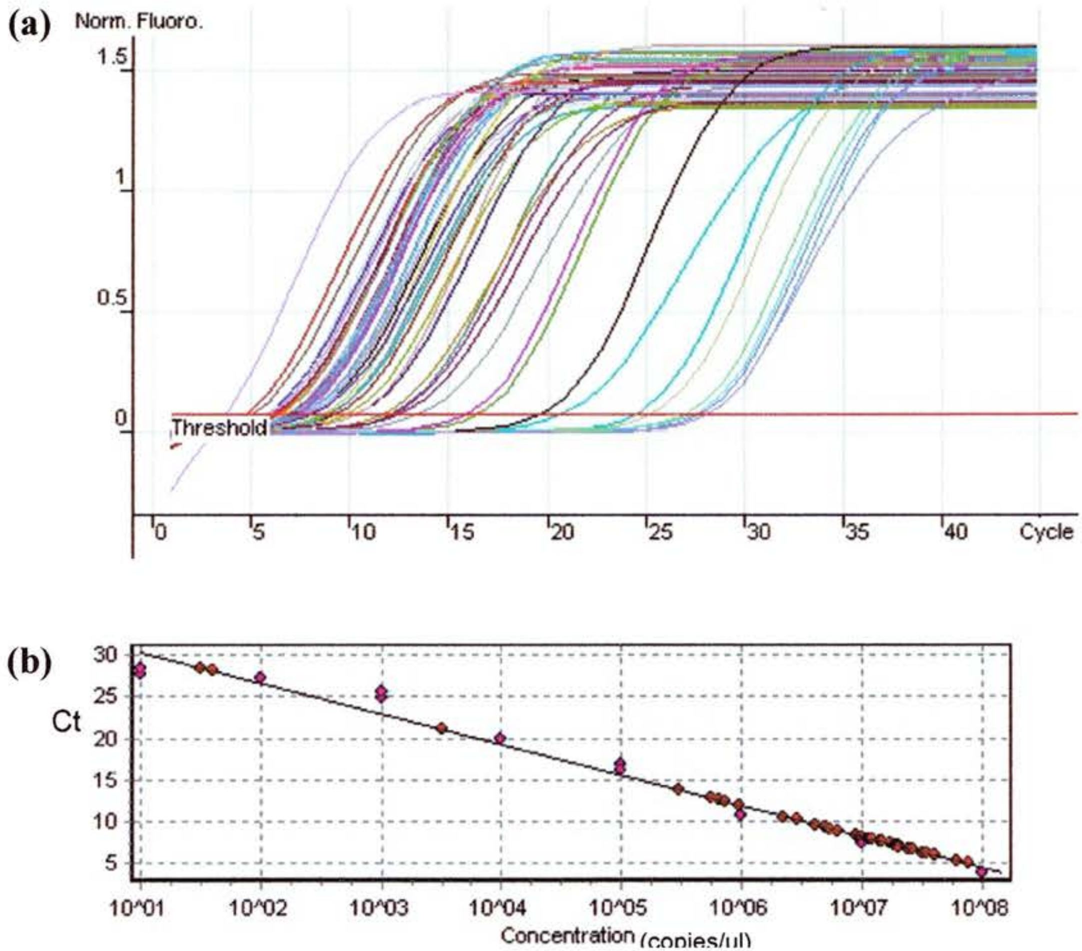


Figure 6.13 Detection and quantification of TGF-β mRNA by real-time RT-PCR

Normalised fluorescence intensities were plotted against PCR cycle number (a). The horizontal red line indicates the fluorescence threshold of amplification detection at normalised fluorescence = 0.052, set within the exponential phase and determined by real-time software. Absolute copy numbers of unknown samples (dots in red) were derived from data of standards (dots in blue) (b). The value of Ct ranges from 27.150 to 42.580 with a mean of 32.352. The copy number per μl ranges from 3,783 to 0 with a mean of 941.

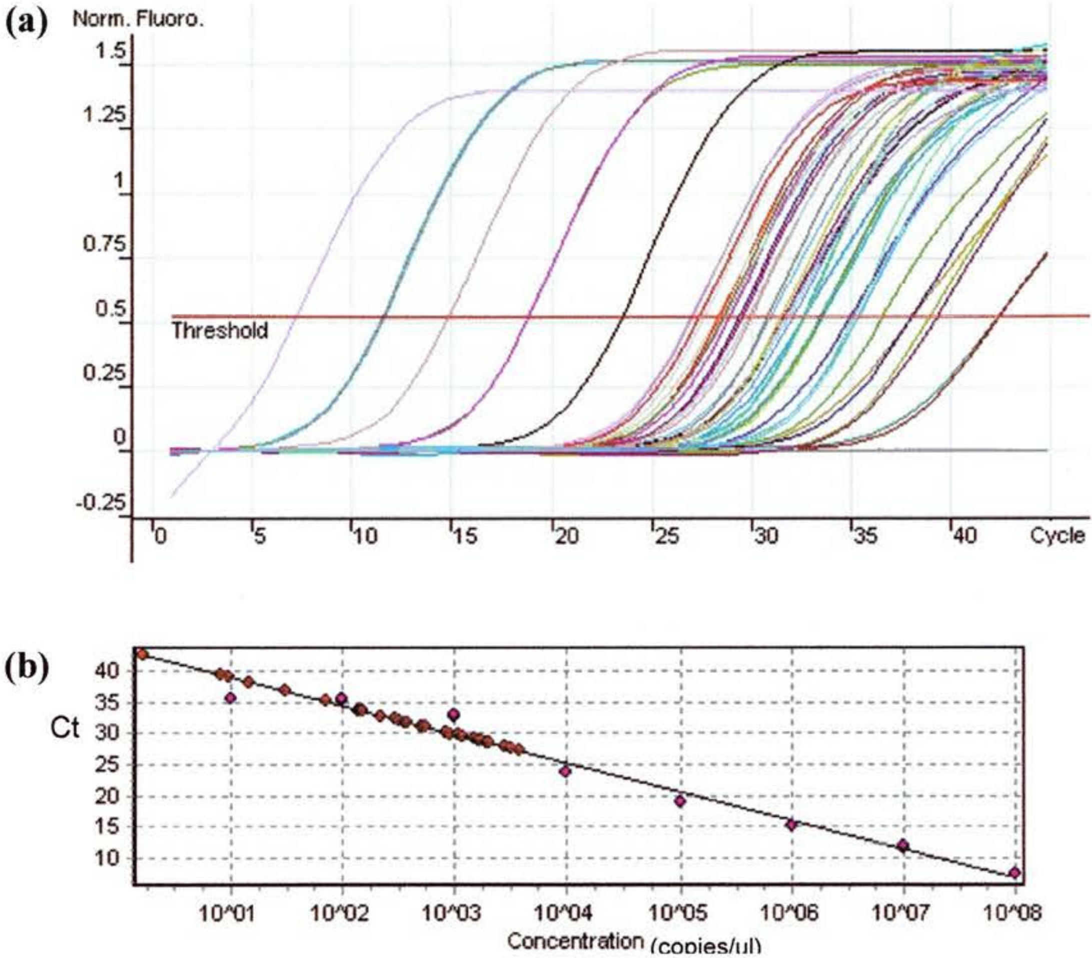


Figure 6.14 Detection and quantification of IL-4 mRNA by real-time RT-PCR

Normalised fluorescence intensities were plotted against PCR cycle number (a). The horizontal red line indicates the fluorescence threshold of amplification detection at normalised fluorescence = 0.2844, set within the exponential phase and determined by real-time software. Absolute copy numbers of unknown samples (dots in red) were derived from data of standards (dots in blue) (b). The value of Ct ranges from 27.78 to 41.04 with a mean of 34.329. The copy number per µl ranges from 521 to 0 with a mean of 50.4.

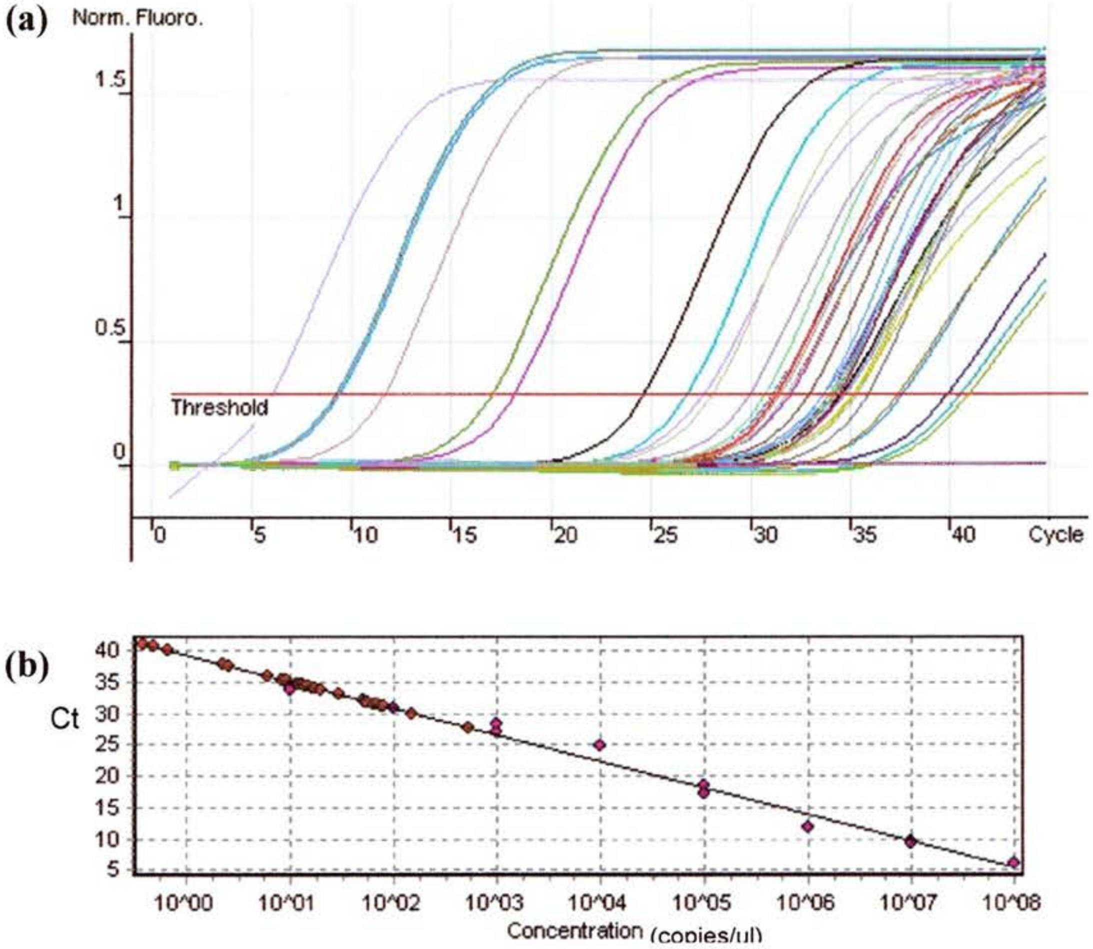


Figure 6.15 Detection and quantification of IFN-γ mRNA by real-time RT-PCR

Normalised fluorescence intensities were plotted against PCR cycle number (a). The horizontal red line indicates the fluorescence threshold of amplification detection at normalised fluorescence = 0.0164, set within the exponential phase and determined by real-time software. Absolute copy numbers of unknown samples (dots in red) were derived from data of standards (dots in blue) (b). The value of Ct ranges from 28.748 to 36.06 with a mean of 30.203. The copy number per µl ranges from 150 to 0 with a mean of 19.

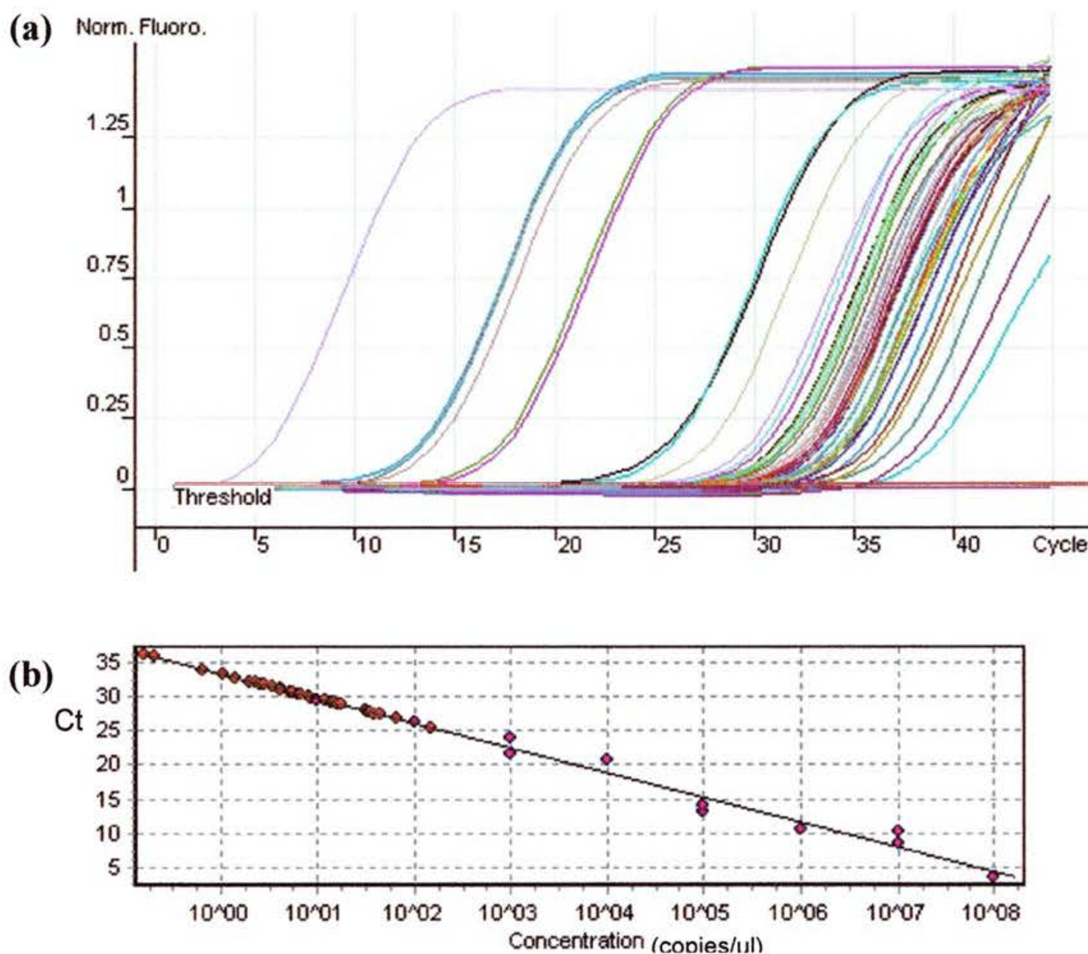


Figure 6.16 Detection and quantification of iNOS mRNA by real-time RT-PCR

Normalised fluorescence intensities were plotted against PCR cycle number (a). The horizontal red line indicates the fluorescence threshold of amplification detection at normalised fluorescence = 0.0152, set within the exponential phase and determined by real-time software. Absolute copy numbers of unknown samples (dots in red) were derived from data of standards (dots in blue) (b). The value of Ct ranges from 24.79 to 35.68 with a mean of 27.068. The copy number per μl ranges from 429 to 0 with a mean of 85.5.

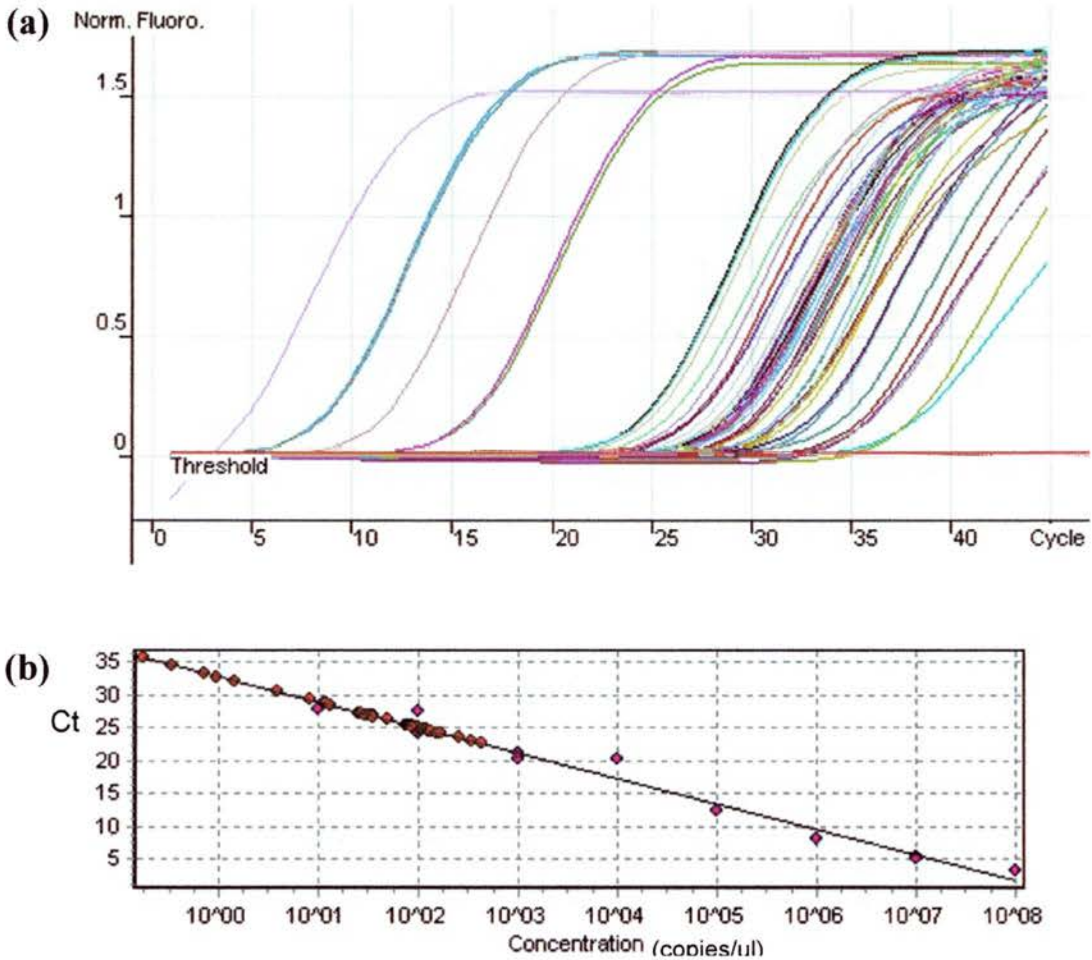


Table 6.3 Undetected mRNA transcript expressions in samples

	18S rRNA	TGF-β	IL-4	IFN-γ	iNOS
Undetected	0	5	7	3	0
Lesional (n=5)	0	1	3	1	1
Non-Lesional (n=20)	0	3	3	2	1
Healthy (n=10)	0	1	3	2	0

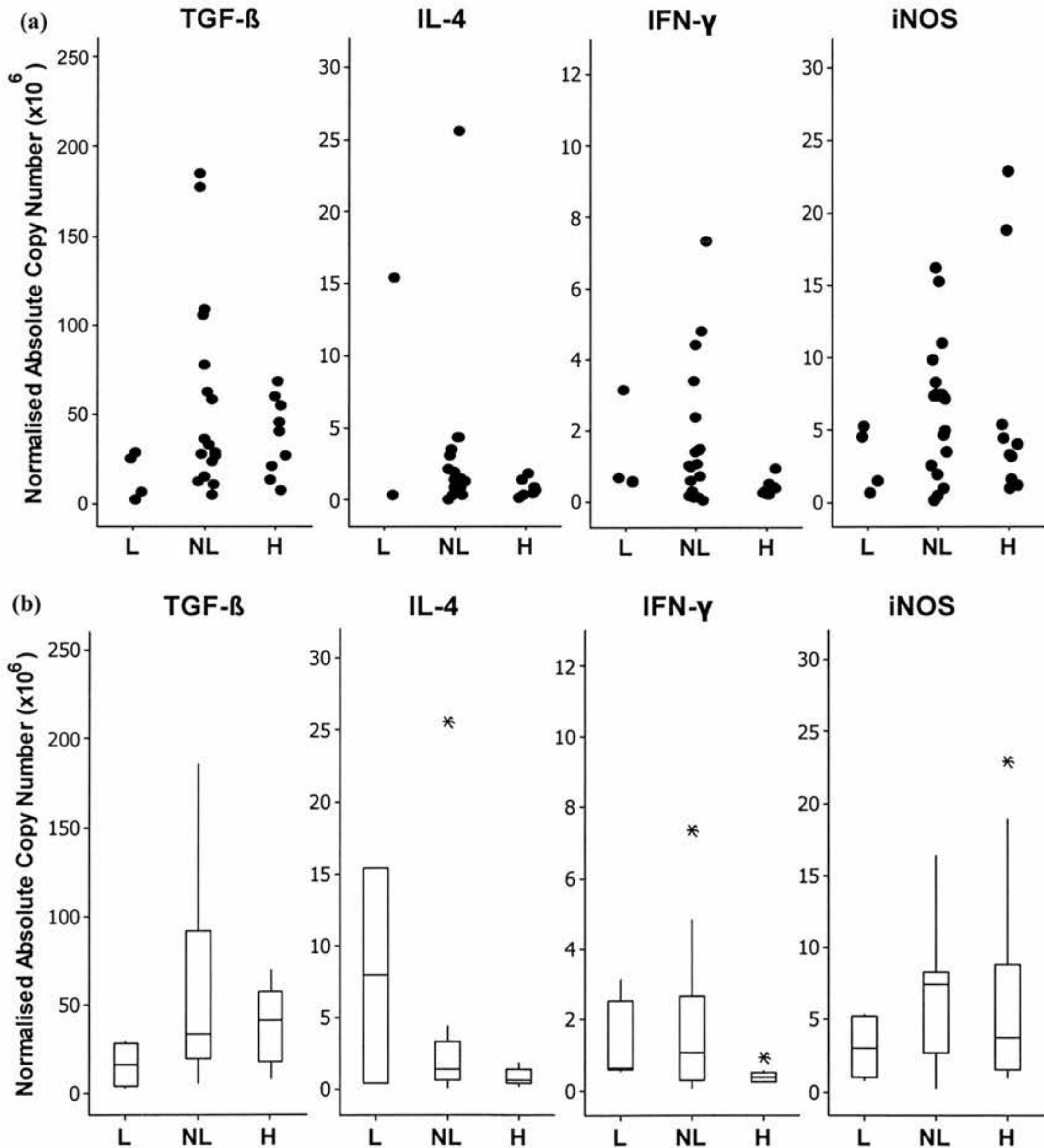
Table 6.4 Descriptive statistics of normalised absolute copy numbers

Variable	N	N*	Mean	SE Mean	St Dev	Minimum	Q1	Median	Q3	Maximum
TGF-β										
L	4	1	16.19	6.60	13.20	3.08	3.99	16.28	28.31	29.12
NL	17	3	58.9	13.5	55.6	5.78	19.7	33.0	91.8	185.7
H	9	1	38.09	7.16	21.47	7.90	17.72	41.22	57.68	69.12
IL-4										
L	2	3	7.94	7.50	10.60	0.447	-	7.94	-	15.44
NL	17	3	3.16	1.44	5.93	0.117	0.673	1.41	3.31	25.57
H	7	3	0.851	0.224	0.592	0.232	0.397	0.668	1.401	1.869
IFN-γ										
L	4	1	1.237	0.632	1.264	0.546	0.556	0.635	2.520	3.131
NL	18	2	1.770	0.472	2.002	0.0583	0.254	1.048	2.649	7.363
H	8	2	0.4088	0.0845	0.239	0.2106	0.2312	0.3539	0.5024	0.934
iNOS										
L	4	1	3.06	1.12	2.24	0.782	0.968	3.04	5.16	5.37
NL	19	1	6.58	1.04	4.52	0.239	2.64	7.40	8.30	16.29
H	10	0	6.63	2.44	7.71	1.00	1.54	3.73	8.80	22.93

N: number of samples; N*: number of missing values; SE mean: standard error of mean; St Dev: standard deviation; Q1: 25th quartile; Q3: 75th quartile; L: lesional; NL: non-lesional; H: healthy.

Figure 6.17 Cytokine expression measured by qRT-PCR

Absolute copy numbers of TGF-β, IL-4, IFN-γ and iNOS mRNA transcripts normalised against 18S rRNA by dividing target copy number by 18S rRNA copy number and multiply by a factor of 1,000,000 in the three groups of skin samples: lesional atopic skin (L), non-lesional atopic skin (NL) and healthy controls (H) are shown as a scatter plot (a) and box plot (b). The box represents the 25th and 75th quartiles with the horizontal bar indicating the median. The whiskers indicate the range, and * represents outliers.



6.4 Discussion

6.4.1 Quantification of mRNA expression

Four methods are in common use for the quantification of transcription: Northern blotting, *in situ* hybridisation, RNAse protection assays and reverse transcription polymerase chain reaction (RT-PCR). A fifth method, cDNA arrays, is still limited in its use by cost considerations. RT-PCR is an *in vitro* method for enzymatically amplifying defined sequences of RNA and permits the analysis of samples containing very little starting material. It is the most exquisitely sensitive and the most flexible of the quantification methods and can be used to compare the levels of mRNAs in different sample populations, to characterise patterns of mRNA expression, and to discriminate between closely related mRNAs (Bustin 2000).

Any PCR reaction can be divided into four different kinetic stages (Freeman and others 1999; Wilhelm and Pingoud 2003). First, there is a lag phase where exponential amplification is already ongoing within the PCR tube, but no fluorescence signal above the background level is measurable. Secondly, in the logarithmic (log) phase the exponential growth of PCR product – ideally, there is a doubling of PCR product every cycle – is measurable as fluorescent signal. Thirdly, in the retardation phase, accumulation of PCR inhibiting factors and loss of enzyme and substrates for the PCR decelerates the reaction. Fourthly, the PCR reaches a steady state in the stationary phase, and no more amplicons are produced (Pannetier and others 1993; Freeman and others 1999). Theoretically, during the exponential growth phase, there is a quantitative relationship between amount of starting target sequence and amount of PCR product at any given cycle, and a linear relationship exists between the number of amplification cycles and the logarithm of the number of molecules. However, the efficiency of amplification is decreased because of contaminants (inhibitors), competitive reactions, substrate exhaustion, inactivation of the polymerase and target reannealing. As the number of cycles increases, the amplification efficiency decreases, eventually resulting in a plateau effect.

The conventional RT-PCR utilising end-point product analysis is not able to determine the initial quantity of template molecules for a gene-specific PCR, because

at the end of the amplification cycles the amount of amplicon depends not only on the input amount but also technical variations occurring during the reaction (Liu and Saint 2002). Quantitative PCR requires the measurement to be taken prior to the reaction plateau. Some applications are designed to include an endogenous internal standard, i.e. housekeeping gene, to monitor the efficiency of amplification. Amplification of housekeeping genes verifies that the target nucleic acid and reaction components were of acceptable quality but does not account for differences in amplification efficiencies due to differences in product size or primer annealing efficiency between the internal standard and target being quantified.

Analysis of PCR product by gel electrophoresis can be at best semi-quantitative because amplification efficiencies are not known. Real-time PCR instruments detect amplicons as they accumulate, by measuring an increase in fluorescence resulting from DNA synthesis (Higuchi and others 1992).

To date, published studies investigating the cytokine expression in dogs with atopic dermatitis have used basic RT-PCR (Olivry and others 1999), semi-quantitative RT-PCR (Hayashiya and others 2002; Nuttall and others 2002a; Nuttall and others 2002b) and relative quantification using real-time RT-PCR (Fujiwara and others 2003). In this study, by cloning, transformation, plasmid preparation, linearisation, verification and exact determination of standard concentration, it was possible to generate very stable and highly reproducible standard curves for each target gene. This information was then used to calculate the absolute copy number of target transcripts. This is the first time the use of such standard curves has been reported in the veterinary literature. This method is superior to conventional RT-PCR and should yield more accurate and reliable data.

When this method was applied to the measurement of cytokines in canine skin, in general, major significant differences were not observed between the different skin groups. As with any biological experiment, the first question is whether or not this is a true biological finding or if it could reflect lack of statistical power or technical issues (false positive or negative results). In this study, the group size in the lesional

skin group was small which would have a negative impact on statistical power. This is always a problem when dealing with samples obtained from clinical cases, especially when such research samples have to be obtained under home office licence and with owner's consent. Obviously, a larger group size would have been desirable but it was not possible due to logistical reasons.

Technical factors also come into play when interpreting data from RT-PCR reactions. In general, real time PCR can only be used to reliably detect a minimum of a two fold difference in DNA levels between samples. This essentially represents one cycle (because the amount of DNA approximately doubles in each cycle). In this study, the use of standard curves and normalisation to a housekeeping gene should have minimised inaccuracies due to technical issues but, despite this, the method would still not have been able to detect more subtle differences between groups if those differences were less than two fold.

A further technical issue relates to the quality of the RNA in the archived samples. Results from the electropherograms indicating that partial degradation of RNA had occurred in some of the samples which might have influenced the final analysis.

Despite the general lack of significant statistical differences, there were some trends in the data that warrant further comment and comparison to existing literature.

6.4.2 Th2 and Th1 cytokine expression in canine atopic dermatitis

Studies in humans and experimental mouse models suggest that IL-4 expression is a hallmark of atopic dermatitis (Leung 2000). No significant difference was found in IL-4 mRNA expression between the groups in our study, but there was a tendency for higher levels to be seen in atopic skin. In Nuttall's study (using the same samples), this difference was significant (Nuttall and others 2002a; Nuttall and others 2002b). In Marsella's study, using genetically predisposed high-IgE Beagles, no increase in IL-4 mRNA was recorded after epi-cutaneous allergen challenge (atopy patch testing). An earlier study using non-quantitative methods found that IL-4 mRNA was expressed in both atopic and healthy skin samples (Olivry and others

1999) whereas in another study using quantitative RT-PCR, IL-4 mRNA was not detected in any of the skin samples from either atopic or healthy dogs examined (Maeda and others 2002). Possible explanations for these discrepancies are discussed at the end of this section.

Studies evaluating IL-4 mRNA expression in PBMCs have showed similar results. Levels of IL-4 in resting PBMCs were similar in both atopic and healthy dogs whereas significantly higher levels of IL-4 mRNA were detected in *D. farinae* stimulated atopic PBMCs compared to healthy PBMCs in a semi-quantitative study using samples from naturally occurring atopic dogs (Nuttall 2003). In another study, the amount of IL-4 mRNA was below the lower detection limit in all of the sensitised and control dogs in primary PBMCs but was markedly increased after stimulation with the sensitising antigen in a quantitative study using experimentally sensitised dogs (Fujiwara and others 2003). These findings suggest that Th2 responses in atopic dogs are the result of interaction with specific allergens rather than the result of global Th2 polarisation. It has also been confirmed in humans that the Th2 responses in atopic patients are allergen specific and are not associated with non-sensitising antigens (Jenmalm and others 2001).

It is likely that other Th2-type cytokines such as IL-13, IL-6 and IL-5 could be associated with Th2 polarisation and IgE production in atopic disease but their expression was not investigated in this study. Further studies should examine a wider range of cytokines over a dynamic period in order to elucidate the complex orchestration of the cytokine milieu in canine atopic dermatitis.

Higher levels of mRNA for the Th1 cytokine IFN-γ were seen in atopic dog skin as compared with healthy controls in this study. This result is consistent with previous findings. Studies using skin samples from naturally occurring atopic dogs have shown that significantly higher levels of IFN-γ mRNA were detected in lesional compared with non-lesional and healthy skin (Maeda and others 2002; Nuttall and others 2002a; Nuttall and others 2002b), and mRNA expression of IFN-γ also

significantly increased during epi-cutaneous allergen challenge in high-IgE Beagles (Marsella and others 2006).

The increase of IFN-γ in atopic group in our study is, however, contrary to other reports (Hayashiya and others 2002; Fujiwara and others 2003) in which the expression of IFN-γ mRNA in primary PBMCs both in naturally occurring AD and in experimentally sensitised dogs was significantly lower than that in the healthy dogs. Several studies in humans have shown an increased frequency of allergen-specific T cells producing little IFN-γ in the peripheral blood and skin lesions of patients with AD (van der Heijden and others 1991; van Reijsen and others 1992). After stimulation with sensitising antigen, the expression level of IFN-γ mRNA in the PBMCs from sensitised dogs was similar to that in PBMCs from the healthy dogs (Fujiwara and others 2003). However, one unpublished report showed that similar levels of IFN-γ mRNA were present in both atopic and healthy dogs in resting PBMCs, but significantly greater levels of IFN-γ transcription were seen in stimulated healthy PBMCs compared to atopic PBMCs (Nuttall 2003). In Nuttall's study, canine immunological responses to the antigens from house dust mite *D. farinae* in naturally occurring atopic dogs were investigated whilst Fujiwara studied the immune responses to antigens from Japanese cedar *Cryptomeria japonica* in experimentally sensitised dogs. It is not known whether different antigens activate different immunological pathways in disease development. Further studies to identify the cells producing these cytokines and to compare the mRNA expression with the protein production are needed to make a complete interpretation.

Th1 and Th2 cytokines contribute to the pathogenesis of skin inflammation in human AD with the relative contribution of each cytokine dependent on the duration of the skin lesion (Leung 2000). Controlled studies evaluating cytokine expression in relation to lesion development would provide more genuine information. It has been shown in humans that acute T-cell infiltration in AD is associated with a predominance of IL-4 and IL-13 expression, whereas maintenance of chronic inflammation is associated with increased IL-5, IL-12 and IFN-γ expression accompanied by the infiltration of eosinophils and macrophages (Hamid and others

1994; Hamid and others 1996). The biphasic pattern of T-cell activation has also been demonstrated in studies on allergen patch testing (Grewe and others 1998). Increased expression of IL-4 mRNA and protein is observed 24 hours after allergen application to the skin, after which IL-4 expression declines to baseline levels. In contrast, IFN-γ mRNA expression is not detected in 24-hour patch-test lesions but is strongly over-expressed at the 48- and 72-hour time points (Grewe and others 1998). Atopy patch testing in dogs has recently been performed (Marsella and others 2006) and the authors concluded that early lesions are overall associated with high transcription and translation of pro-inflammatory cytokines (e.g. IL-6) and Th2 cytokines (e.g. IL-13), while later lesions are accompanied by progressive increase of Th1 cytokines (e.g. IL-18) (Marsella and others 2006). In the same study, IFN-γ mRNA showed a biphasic response with an early peak at 6-hour; the early rise of INF-γ could be potentially explained by the presence of some T-cell infiltration even prior to any allergen challenge, as indicated by the presence of a mononuclear infiltrate in both baseline and 6 h biopsies (Marsella and others 2006).

Th1 cytokines appear to be important in the establishment and maintenance of chronic lesions, but the trigger for Th1 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 2001). However, clinically uncomplicated canine atopic dermatitis is largely a pruritic and erythematous disease and most lesions result from self-trauma and microbial colonisation (Rothe and Grant-Kels 1996; Griffin and DeBoer 2001a). Self-trauma induced by scratching or rubbing severely pruritic skin can induce inflammatory cytokines (e.g. IL-1, TNF-α and GM-CSF) from keratinocytes and result in the recruitment of mononuclear cells and ultimately the development of chronic lesions (Robert and Kupper 1999). Bacterial activation of Toll-like receptors by cutaneous bacteria and increased expression of MHC II, IL-1 and IL-12, up-regulated endothelial ICAM-1 and VCAM-1, as well as increased CLA expression on T-cells induced by staphylococcal superantigens also contribute to the development of chronic skin lesions.

The Th1-type cytokines IL-2, IFN-γ and TNF-α are associated with cell-mediated inflammation and inhibition of Th2 differentiation (Fiorentino and others 1989). APC derived IL-12 is a critical factor in Th1 differentiation, IFN-γ production and CLA expression on circulating T-cells (Snijders and others 1998). Studies using microarray investigating thousands of genes at the same time would provide more information regarding the intricate interaction between various cytokines and cells involved in the pathogenesis of atopic dermatitis.

There are various explanations for the discrepancies and conflicting data in relation to the role of Th2 and Th1 cytokines in canine atopic dermatitis. First, there may be differences due to varying methodologies. Conflicting results relating to different methods used to quantify cytokine gene transcripts have been shown in another study in dogs suffering from chronic diarrhoea. Using basic semi-quantitative RT-PCR, increased concentration of IL-2, IL-5, IL-12p40, TNF-α and TGF-β mRNA transcripts were reported in the duodenal mucosa of German Shepherd dog with chronic enteropathies compared with control dogs (German and others 2000). However, no difference in expression of these cytokines was found in a follow-up study using real-time quantitative RT-PCR (Peters and others 2005). Real-time RT-PCR provides a more accurate representation of tissue mRNA concentration over conventional RT-PCR because the latter does not differentiate exponential amplification and plateau reaction, and therefore the intensity of the gel band may bear little relation to the starting template quantity. Hence, cautious interpretation should be exercised when comparing results from various studies in which different methodologies were employed.

Second, the varying results obtained by different authors who have assessed Th2 and Th1 cytokine profiles in healthy and atopic dogs could be explained by obtaining "snapshots" of the cytokine milieu at a particular point in time, whereas in reality, the situation is in a constant state of flux. As the precise "age" of lesions in clinical cases is not known at the time of biopsy, the particular cytokine in question may or may not be present in abnormal amounts.

Taken together, the balance of evidence from this and previous studies suggests that IL-4 may be increased in the skin of atopic dogs throughout the pathological continuum, but its level may be very variable depending on the exact age of the lesion and the particular cyto-humoral milieu at a particular point in time. It has been reported in the human literature that IL-13 rather than IL-4 is released by peripheral T cells of patients with AD and in lesional skin (Akdis and others 1999b; Aleksza and others 2002). In contrast, IFN-γ appears to increase as the chronic inflammatory reaction develops. Unfortunately, due to the limited availability of antibodies suitable for immunohistochemical staining of cytokines, all studies investigating the cytokine milieu in atopic dogs have so far had to use PCR techniques. In the future, immunohistochemistry and proteomic analysis might confirm whether or not there is genuine over-expression of these specific proteins in atopic canine skin.

6.4.3 Immunosuppressive cytokines in canine atopic dermatitis

The TGF-β family of proteins are a set of pleiotropic secreted signalling molecules with unique and potent immuno-regulatory properties. TGF-β is known to affect T cell proliferation, differentiation, apoptosis, antigen presentation, effector functions of macrophages, the expression of MHC I, MHC II, CD40 and IL-12 (Jutel and others 2003). It can also modulate expression of adhesion molecules, provide a chemotactic gradient for leukocytes and other cells participating in an inflammatory response, and inhibit them once they have become activated (Letterio and Roberts 1998). However, the role TGF-β plays in the canine immune system is less well understood.

In this study, higher levels of TGF-β mRNA were detected in healthy and non-lesional skin compared to lesional atopic skin. These results are in general agreement to those of Nuttall who used the same tissue samples, although in his study, lower levels of TGF-β mRNA were found in both non-lesional and lesional atopic skin (Nuttall and others 2002b). These results suggest that the expression of

TGF- β might be down-regulated in lesional atopic skin, and the lack of its immunosuppressive effect might be one factor that allows the inflammatory reaction to progress. .

TGF-β, and IL-10, mediate certain aspects of peripheral immune tolerance to environmental allergens. Both $CD4^+$ and $CD8^+$ suppressive T cell subsets that secrete active TGF-β have been identified following induction of oral tolerance (Chen and others 1995), and the protective function of these cells both *in vitro* and *in vivo* can be blocked by antibodies to TGF-β (Chen and others 1994). Furthermore, TGF-β and IL-10 have been shown to cooperate in suppression of the immune response to aeroallergens and control allergic inflammation due to allergen exposure in healthy individuals as well as in allergen-specific immunotherapy for house dust mite allergic patients (Jutel and others 2003). The low levels of cutaneous TGF-β mRNA in lesional skin found in this study could, therefore, reflect a breakdown of suppressive mechanisms. In contrast, constitutive expression of TGF-β in non-lesional and healthy skin could inhibit inappropriate inflammatory responses to environmental allergens. The balance of anti-inflammatory and stimulatory activity of TGF-β may 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)

6.4.4 Inducible nitric oxide synthase expression

Nitric oxide (NO) is a highly diffusible intercellular signalling molecule involved in a wide range of biological effects (Anggard 1994). It is generated by the enzyme nitric oxide synthase (NOS), and three genetically distinct NOS isoforms have been characterised in humans. Two isoforms are constitutively expressed (cNOS, including endothelial NOS or eNOS and neuronal NOS or nNOS), whereas one isoform is an inducible enzyme (iNOS, NOS2). The cNOS isoforms are active only after binding of calcium-calmodulin complexes and produce NO for short periods of time (seconds to minutes), and NO synthesised by the cNOS isoforms usually acts as an intercellular signalling molecule mediating time-restricted events such as neurotransmission or vasorelaxation (Lowenstein and Snyder 1992; Knowles and

Moncada 1994). It has been suggested that the cNOS pathway provides regulatory and homeostatic functions in the skin (Bruch-Gerharz and others 1998). In contrast to the cNOS isoforms, iNOS is transcriptionally regulated and requires *de novo* messenger RNA and protein synthesis for its activity (Kroncke and others 1995). Once induced, iNOS is active for comparatively long periods of time (hours to days), thereby producing large amounts of NO, which can display non-specific host protective functions and immuno-regulatory and cytotoxic activities (Liew and Cox 1991; Nathan 1992). It has been shown that the transcriptionally regulated induction of iNOS can be inhibited by glucocorticosteroids, cyclophilins, and retinoids as well as TGF-β, IL-4 and IL-10 (Becherel and others 1996). It has been found in most cells of the skin, including keratinocytes, Langerhans cells, fibroblasts and endothelial cells (Bruch-Gerharz and others 1998). Research from experiments with mice lacking a functional iNOS gene showed that NO generated from iNOS activity may also be involved in the regulation of Th1 cell population expansion and Th1-mediated immune responses (MacMicking and others 1995; Wei and others 1995). The expression of iNOS in cells of the skin is predominantly associated with inflammatory processes early in cutaneous immune responses (Bruch-Gerharz and others 1998).

Using immunohistochemistry, eNOS and iNOS have been measured in skin from human patients with AD, allergic contact dermatitis (CD) and psoriasis (Rowe and others 1994; Rowe and others 1997). Expression of iNOS mRNA was found to be decreased in AD, as compared with psoriasis, skin using GeneChip microarrays (Nomura and others 2003), but the immunoreactivity of iNOS was significantly more extensive in AD skin than in CD skin using immunohistochemistry (Rowe and others 1997). Induction of iNOS in the dermal endothelium and in perivascular inflammatory cells may be significant with respect to the roles of NO in both the vasodilatory component of the inflammatory response and in the modulation of immune responses in the skin (Rowe and others 1997). However, the potential role of iNOS in the context of AD is not fully understood.

In this study, the expression of iNOS mRNA transcripts was lower in lesional compared with non-lesional skin samples from atopic dogs although the difference did not reach statistical significance. These results were unexpected. The primers used in RT-PCR were designed to specifically amplify transcripts of iNOS, and amplification of neuronal NOS (NOS1) or endothelial NOS (NOS3) was not likely, if not impossible.

It has been shown that iNOS expression is found in a number of inflammatory skin conditions including psoriasis, atopic dermatitis, irritant and allergic contact dermatitis (Kolb-Bachofen and others 1994; Rowe and others 1997; Ormerod and others 1997; Ormerod and others 1998). The mRNA or protein levels of iNOS are usually the highest in lesional skin followed by clinically uninvolved skin, and the expression of iNOS is often minimal in healthy skin (Rowe and others 1997; Ormerod and others 1998). iNOS is not usually found in normal resting cells, but induced in response to inflammatory cytokines or a combination of cytokines (i.e. IFN-γ, IL-1β, IL-2, IL-8 and TNF-α) and bacterial polysaccharides (Cals-Grierson and Ormerod 2004). However, these cytokines are either absent or only present at low levels in AD lesions, and some of the cytokines that are present (such as IL-4) may have inhibitory effects on iNOS expression. It is possible that the cytokine milieu in AD prevents the induction of iNOS due to lower levels of pro-inflammatory cytokines such as TNF-α or IFN-γ and the increased Th2 cytokines (Nomura and others 2003).

In summary, the levels of iNOS mRNA in canine atopic skin appeared to be lower than in healthy skin. As this is a paradoxical finding, it needs to be verified in future studies.

Chapter 7

Conclusion

7.1 D. farinae-specific IgG responses in canine atopic dermatitis

This thesis describes the immunological features of dogs with 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 role of IgG is not clear and remains paradoxical. The most common allergens are the house dust mites *Dermatophagoides farinae* and *D. pteronyssinus*, but the major allergen for atopic dogs is a 98-kDa chitinase (Der f 15) rather than group 1 or 2 allergens (Noli and others 1996; McCall and others 2001). By developing and validating a digital image analysis method for Western blots, we were able to study and semi-quantify global antibody responses against various *D. farinae* antigens simultaneously without operator's bias. The technique has its superiority over existing antibody study methods, i.e. ELISA, in that comprehensive antigen-specific antibody response can be investigated and distinguished contemporaneously. The methodology we developed provides a powerful surveillance capacity in identifying major antigens of crude allergen extract when studying novel allergens. It is also ideal for monitoring different specific antibody responses in allergic patients undergoing treatment, i.e. ASIT, and should be used in initial investigation stage identifying potential target antigens responsible for successful therapy.

The profile of IgG binding against *D. farinae* antigens was similar in dogs with atopic dermatitis and healthy dogs, and the responses were generally stronger in atopic dogs although the specific antibody levels did not differ significantly. The generation of specific antibody response to ubiquitous environmental allergen seem to merely represent the recognisition of foreign antigen by canine immune system, and a reciprocal interaction of other factors including predisposed genetic

background, early immunisation of the offending allergen, vigorous vaccination programme, etc has a prominent influence on the development of atopic disease than the downstream antibody response.

The *D. farinae*-specific IgG responses were consisted predominantly of the subclass IgG1 and IgG4, and *D. farinae* specific IgG2 and IgG3 were not detected in atopic dogs. This finding is in consistent with the previous study showing a degree of IgG subclass restriction in the humoral immune response of canine atopy which may be dependent upon the nature of the allergen (Day and others 1996). However, the function of dog IgG subclass antibodies has not been examined due to limited availability of suitable specific reagents, and further study is warranted to establish the functionality of IgG subclasses in the context of allergic disease.

Taken together, the results of these studies suggest that *D. farinae*-specific IgG and IgG subclasses do not play a major role in the pathogenesis of canine atopic dermatitis. Such responses occur in both healthy and atopic dogs and do not appear to be either protective or pathogenic. These findings would fit with a hypothesis in which the production of IgG, and its subclasses, against *D. farinae* antigens was merely a result of recognition of a foreign protein that was presented to the immune system in relatively large quantities. This would also fit with the concept of *D. farinae* antigens acting as 'danger' signals to the immune system, thus provoking an antibody response even though the molecules themselves appear to be harmless. In view of this, it seems likely that the production of IgE and the activation of allergen-specific T lymphocytes are the most important immunological events in canine atopic dermatitis.

7.2 D. farinae-specific IgG responses in atopic dogs undergoing allergen-specific immunotherapy

In atopic dogs undergoing allergen-specific immunotherapy using alum-precipitated vaccines or aqueous vaccines, *D. farinae*-specific IgG responses were also investigated. Whilst specific antibody responses did not change in relation to the outcome of treatment or dosage of vaccine in atopic dogs using alum-precipitated

vaccines, *D. farinae*-specific IgG levels were shown to increase significantly after 2 month of ASIT using aqueous vaccines. If specific IgG antibody was not induced after ASIT, the beneficial effect of successful ASIT might be accounted for by other mechanism such as down-regulation of cellular function, induction of regulatory T cells or changes in immuno-suppressive/immuno-regulatory cytokine profile. On the other hand, the study using aqueous vaccines demonstrated a dramatic increase in *D. farinae*-specific IgG level, and supported the hypothesis of blocking antibody theory. Although clinical improvement did not correspond well with *D. farinae*-specific IgG response because most of the atopic dogs included in the study were sensitised to multiple allergens in addition to house dust mite, the induction of IgG antibody demonstrated a possible role of IgG in the pathogenesis of canine atopic dermatitis. Further studies comparing the *D. farinae*-specific IgG response to clinical outcome in dogs that are mono-sensitised to dust mite allergen would yield an explicit picture of the role of *D. farinae*-specific IgG.

A potentially exciting finding was noticed in that atopic dog with a pre-existing high level of IgG antibodies to *D. farinae* prior to the onset of ASIT was less likely to respond to the treatment. This may suggest a means by which dogs could be screened prior to initiation of ASIT; alternatively, this might allow alterations in dosage schedule to be predicted in advance. Additional methods are required to substantiate this finding in a larger group of dogs. If this is a repeatable phenomenon, *D. farinae*-specific IgG level could be used as a prognostic indicator in clinical settings to assist the assessment of whether to engage ASIT.

7.3 Cytokine responses in canine atopic dermatitis

Upon exposure to most foreign antigens, the usual humoral immune response results in production of IgG antibody rather than IgE. A major determinant of which antibody class predominates is which one of the two subsets of helper T lymphocytes (Th1 and Th2) is dominant. The factors that determine whether a Th1 or a Th2 response will predominate are complex but include both genetic and environmental influences.

Previous studies using semi-quantitative RT-PCR had demonstrated that the skin of dogs with AD over expressed IL-4 mRNA and under expressed TGF-β compared with healthy dog skin, which indicated a Th2-biased response (Nuttall and others 2002b). We investigated the four arms of immune responses – the Th1 type, the Th2 type, the regulatory and the innate immune responses – by studying one representing cytokine for each response using quantitative real-time RT-PCR. The system was validated using plasmid DNA constructs containing the sequences of interest, and the generation of absolute quantity of target gene mRNA transcripts normalised to endogenous internal control 18S rRNA provided superior quantitative accuracy as compared with the conventional RT-PCR techniques. The immunoregulatory cytokine TGF-β tended to be suppressed in lesional skin samples, whilst the innate immune response cytokine iNOS expression was somehow lower in healthy skin as compared to atopic skin. IL-4 was not significantly expressed in the skin of atopic dogs throughout the pathological continuum, whereas IFN-γ appeared to increase as the chronic inflammatory reaction developed.

It is interesting to note that the level of IL-4 mRNA transcripts in atopic dog skin and primary PBMC was often below the detection limit in real-time RT-PCR despite its supreme sensitivity (Maeda and others 2002; Fjuiwara and others 2003; Marsella and others 2006). Also, statistical significant differences in mRNA expression were not often reached using real-time RT-PCR methodology as compared with semi-quantitative RT-PCR in studying dog with bowel disease (German and others 2000; Peters and others 2005), as well as in our study. The biological meaning of such finding remains to be determined. Future cytokine profile studies should combine with immunohistochemistry, proteometic and microarray analysis, and also to investigate a lesion age-related cytokine milieu to characterise potential dynamic and sequential activation of different T cell subsets. Advances in our understanding of this complex disease will no doubt continue to provide us with additional therapeutic solutions in the future.

7.4 Future studies

In order to understand why allergen-specific immunotherapy benefits some atopic dogs but not others, it is necessary to elucidate how ASIT modulates the immune system. *D. farinae*-sensitised atopic dogs with a pre-existing high level of *D. farinae*-specific IgG antibody prior to ASIT responded poorly to the treatment. Further studies are required to confirm this finding in a larger group of dogs, and to evaluate whether other isotypes of allergen-specific antibody are potential prognostic indicators. Allergen-specific IgE has been used for diagnostic purpose in canine atopic dermatitis despite poor assay specificity, and *D. farinae*-specific IgE was more prevalent in normal dogs than in atopic dogs. Semi-quantitative analysis of *D. farinae*-specific IgE in normal and atopic dogs using immunoblotting technique should be carried out to clarify its function. The role of blocking antibody needs to be pursued further because significant augmentation of *D. farinae*-specific IgG was recorded after ASIT using an aqueous vaccine, although the level of specific IgG did not correlate with clinical outcome. Epitope specificity of IgG antibody also needs to be investigated because it could be one of the missing puzzles in the mechanism of ASIT. In addition, further cytokine response studies are also required and studies should combine cytokine expression experiments with immunohistochemistry, proteomic and microarray analysis in order to identify the key functioning cells and/or cytokines in the complex interaction of various immune components. A thorough understanding of ASIT will hopefully provide us new therapeutic options to control or to cure atopic problems and make a pruritus-free life possible for our loyal caine companion.

Appendix

Raw data of 18S rRNA quantification using qRT-PCR

Name	Type	Ct	Given Conc. (copies/µl)	Calc Conc. (copies/µl)
18S 10^8	Standard	5.82	100000000	92052925
18S 10^8	Standard	5.75	100000000	96008355
18S 10^6	Standard	12.28	1000000	1129371
18S 10^6	Standard	12.45	1000000	1007881
18S 10^4	Standard	19.36	10000	9141
18S 10^4	Standard	18.93	10000	12232
18S 10^2	Standard	26.66	100	64
18S 10^2	Standard	25.50	100	140
NL1	Non-lesional skin	6.78		24385644
NL2	Non-lesional skin	6.78		24385644
NL3	Non-lesional skin	6.01		39557785
NL4	Non-lesional skin	6.37		31631510
NL5	Non-lesional skin	7.07		20372447
NL6	Non-lesional skin	7.35		17141700
NL7	Non-lesional skin	12.41		719625
NL8	Non-lesional skin	7.60		14574054
NL10	Non-lesional skin	7.67		13978857
NL11	Non-lesional skin	8.22		9910283
NL12	Non-lesional skin	21.07		3190
NL13	Non-lesional skin	8.01		11323225
NL14	Non-lesional skin	6.66		26337178
NL15	Non-lesional skin	7.98		11501821
NL16	Non-lesional skin	7.22		18546602
NL17	Non-lesional skin	7.88		12295082
NL18	Non-lesional skin	8.24		9808800
NL19	Non-lesional skin	6.24		34303139
NL20	Non-lesional skin	9.60		4182282
NL21	Non-lesional skin	11.94		964345
L1	Lesional skin	10.17		2922969
L2	Lesional skin	9.16		5492067
L3	Lesional skin	9.28		5116187
L4	Lesional skin	10.60		2235485
L5	Lesional skin	12.56		655405
N1	Healthy skin	7.14		19448309
N2	Healthy skin	5.99		40131101
N3	Healthy skin	7.09		20165948
N4	Healthy skin	13.82		298694
N5	Healthy skin	6.69		25881970
N6	Healthy skin	5.29		61944595
N7	Healthy skin	4.97		75984061
N8	Healthy skin	8.91		6423797
N9	Healthy skin	8.40		8863361
N10	Healthy skin	12.79		567033
water	Control			

Given Conc.: given concentration; Calc Conc.: calculated concentration.

Raw data of TGF-β quantification using qRT-PCR

Name	Type	Ct	Given Conc. (copies/μl)	Calc Conc. (copies/μl)
TGF-β 10^8	Standard	5.80	100000000	103482053
TGF-β 10^8	Standard	5.96	100000000	92442478
TGF-β 10^6	Standard	12.67	1000000	1026606
TGF-β 10^6	Standard	12.46	1000000	1183095
TGF-β 10^4	Standard	19.61	10000	9702
TGF-β 10^4	Standard	19.90	10000	7981
TGF-β 10^2	Standard	26.44	100	99
TGF-β 10^2	Standard	26.26	100	112
NL1	Non-lesional skin	28.52		1900
NL2	Non-lesional skin	33.71		141
NL3	Non-lesional skin	29.52		1153
NL4	Non-lesional skin	31.80		368
NL5	Non-lesional skin	27.15		3783
NL6	Non-lesional skin	32.81		222
NL7	Non-lesional skin	42.55		2
NL8	Non-lesional skin	30.13		850
NL10	Non-lesional skin	29.02		1478
NL11	Non-lesional skin	32.04		327
NL12	Non-lesional skin			
NL13	Non-lesional skin	28.41		2009
NL14	Non-lesional skin	28.80		1652
NL15	Non-lesional skin	32.04		327
NL16	Non-lesional skin	31.14		512
NL17	Non-lesional skin	32.26		292
NL18	Non-lesional skin	31.84		361
NL19	Non-lesional skin	27.16		3761
NL20	Non-lesional skin	42.58		2
NL21	Non-lesional skin	38.24		15
L1	Lesional skin	39.12		9
L2	Lesional skin	33.70		142
L3	Lesional skin	33.61		149
L4	Lesional skin	38.23		15
L5	Lesional skin			
N1	Healthy skin	29.66		1075
N2	Healthy skin	27.77		2774
N3	Healthy skin	29.96		927
N4	Healthy skin			
N5	Healthy skin	30.99		552
N6	Healthy skin	28.72		1720
N7	Healthy skin	27.52		3132
N8	Healthy skin	31.70		386
N9	Healthy skin	35.12		70
N10	Healthy skin	39.43		8
water	Control			

Given Conc.: given concentration; Calc Conc.: calculated concentration.

Raw data of IL-4 quantification using qRT-PCR

Name	Type	Ct	Given Conc. (copies/µl)	Calc Conc. (copies/µl)
IL-4 10^8	Standard	5.66	100000000	81050289
IL-4 10^8	Standard	5.59	100000000	85201561
IL-4 10^6	Standard	11.80	1000000	1257116
IL-4 10^6	Standard	11.67	1000000	1377034
IL-4 10^4	Standard	19.11	10000	8780
IL-4 10^4	Standard	18.71	10000	11541
IL-4 10^2	Standard	26.21	100	71
IL-4 10^2	Standard	25.48	100	117
NL1	Non-lesional skin	31.94		54
NL2	Non-lesional skin	35.28		9
NL3	Non-lesional skin	34.08		17
NL4	Non-lesional skin	34.71		12
NL5	Non-lesional skin	27.78		521
NL6	Non-lesional skin	37.79		2
NL7	Non-lesional skin			
NL8	Non-lesional skin	31.64		64
NL10	Non-lesional skin	32.11		49
NL11	Non-lesional skin	34.61		13
NL12	Non-lesional skin			
NL13	Non-lesional skin	34.17		16
NL14	Non-lesional skin	31.96		53
NL15	Non-lesional skin	34.80		11
NL16	Non-lesional skin	34.06		17
NL17	Non-lesional skin	33.82		19
NL18	Non-lesional skin	34.84		11
NL19	Non-lesional skin	30.07		150
NL20	Non-lesional skin			
NL21	Non-lesional skin	37.54		3
L1	Lesional skin	41.04		
L2	Lesional skin	40.67		
L3	Lesional skin	31.25		79
L4	Lesional skin	40.02		1
L5	Lesional skin			
N1	Healthy skin	34.52		13
N2	Healthy skin	31.33		75
N3	Healthy skin	35.41		8
N4	Healthy skin			
N5	Healthy skin	36.00		6
N6	Healthy skin	33.04		30
N7	Healthy skin	31.48		69
N8	Healthy skin	35.26		9
N9	Healthy skin			
N10	Healthy skin			
water	Control			

Given Conc.: given concentration; Calc Conc.: calculated concentration

Raw data of IFN-γ quantification using qRT-PCR

Name	Type	Ct	Given Conc. (copies/µl)	Calc Conc. (copies/µl)
IFN-γ 10^8	Standard	7.48	100000000	94764659
IFN-γ 10^8	Standard	7.51	100000000	93373222
IFN-γ 10^6	Standard	14.31	1000000	1029381
IFN-γ 10^6	Standard	14.26	1000000	1063273
IFN-γ 10^4	Standard	21.03	10000	11925
IFN-γ 10^4	Standard	21.28	10000	10104
IFN-γ 10^2	Standard	28.14	100	108
IFN-γ 10^2	Standard	28.59	100	80
NL1	Non-lesional skin	30.95		4
NL2	Non-lesional skin	29.01		15
NL3	Non-lesional skin	30.29		6
NL4	Non-lesional skin	29.81		9
NL5	Non-lesional skin	25.37		150
NL6	Non-lesional skin	32.65		1
NL7	Non-lesional skin	33.10		1
NL8	Non-lesional skin	27.66		35
NL10	Non-lesional skin	26.64		67
NL11	Non-lesional skin	27.27		44
NL12	Non-lesional skin	36.06		
NL13	Non-lesional skin	29.29		12
NL14	Non-lesional skin	27.46		39
NL15	Non-lesional skin	27.47		39
NL16	Non-lesional skin	28.72		18
NL17	Non-lesional skin			
NL18	Non-lesional skin	29.10		14
NL19	Non-lesional skin	30.98		4
NL20	Non-lesional skin	31.72		3
NL21	Non-lesional skin	33.85		1
L1	Lesional skin	31.89		2
L2	Lesional skin	31.29		3
L3	Lesional skin	31.59		3
L4	Lesional skin	30.19		7
L5	Lesional skin	35.66		
N1	Healthy skin	29.91		8
N2	Healthy skin	28.84		16
N3	Healthy skin	30.60		5
N4	Healthy skin			
N5	Healthy skin	29.92		8
N6	Healthy skin	27.76		33
N7	Healthy skin	28.83		16
N8	Healthy skin	30.52		6
N9	Healthy skin	32.09		2
N10	Healthy skin			
water	Control			

Given Conc.: given concentration; Calc Conc.: calculated concentration.

Raw data of iNOS quantification using qRT-PCR

Name	Type	Ct	Given Conc. (copies/µl)	Calc Conc. (copies/µl)
iNOS 10^8	Standard	6.26	100000000	108789213
iNOS 10^8	Standard	6.31	100000000	105497928
iNOS 10^6	Standard	12.79	1000000	1283984
iNOS 10^6	Standard	12.67	1000000	1393835
iNOS 10^4	Standard	20.49	10000	6800
iNOS 10^4	Standard	21.67	10000	3037
iNOS 10^2	Standard	26.02	100	158
iNOS 10^2	Standard	26.10	100	150
NL1	Non-lesional skin	24.63		121
NL2	Non-lesional skin	28.58		12
NL3	Non-lesional skin	25.36		79
NL4	Non-lesional skin	26.90		32
NL5	Non-lesional skin	24.26		151
NL6	Non-lesional skin	25.31		81
NL7	Non-lesional skin	28.61		11
NL8	Non-lesional skin	24.79		110
NL10	Non-lesional skin	24.87		105
NL11	Non-lesional skin	24.99		98
NL12	Non-lesional skin	34.60		
NL13	Non-lesional skin	25.22		85
NL14	Non-lesional skin	22.50		429
NL15	Non-lesional skin	24.56		127
NL16	Non-lesional skin	26.16		49
NL17	Non-lesional skin	25.18		88
NL18	Non-lesional skin	26.70		35
NL19	Non-lesional skin	23.38		254
NL20	Non-lesional skin	32.15		1
NL21	Non-lesional skin	29.16		8
L1	Lesional skin	35.68		
L2	Lesional skin	27.27		25
L3	Lesional skin	30.48		4
L4	Lesional skin	28.58		12
L5	Lesional skin	33.29		1
N1	Healthy skin	26.85		32
N2	Healthy skin	24.50		131
N3	Healthy skin	25.26		83
N4	Healthy skin	32.80		1
N5	Healthy skin	27.22		26
N6	Healthy skin	25.43		75
N7	Healthy skin	22.90		340
N8	Healthy skin	26.74		35
N9	Healthy skin	24.09		167
N10	Healthy skin	28.37		13
water	Control			

Given Conc.: given concentration; Calc Conc.: calculated concentration.

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Publications arising from this thesis

1. Hou C, Pemberton A, Nuttall T, and Hill PB. (2005) IgG responses to antigens from *Dermatophagoides farinae* in healthy and atopic dogs. Veterinary Immunology and Immunopathology. 106: 121-128.

2. Hou C, Day MJ, Nuttall TJ, and Hill PB. (2006) Evaluation of IgG subclass responses against *Dermatophagoides fairnae* allergens in healthy and atopic dogs. Veterinary Dermatology. 17: 103-110.

3. Hou C, Nuttall TJ, Day MJ, and Hill PB. (2005) *Dermatophagoides farinae*-specific IgG subclass responses in atopic dogs undergoing allergen-specific immunotherapy. In: Hillier A, Foster AP, and Kwochka KW eds. Advances in Veterinary Dermatology, Vol 5. Oxford: Blackwell Publishing. 70-81.

4. Hou C, Griffin CE, and Hill PB. (2007) Changes of *Dermatophagoides farinae*-specific immunoglobulin G in atopic dogs undergoing allergen-specific immunotherapy using aqueous vaccines. Submitted to Veterinary Dermatology.

1. Hou C, Pemberton A, Nuttall T, and Hill PB. (2005) IgG responses to antigens from *Dermatophagoides farinae* in healthy and atopic dogs. Veterinary Immunology and Immunopathology. 106: 121-128.

In this study, we used a semi-quantitative electrophoresis and immunoblotting technique to characterise the IgG response to antigens from *Dermatophagoides farinae* in 20 healthy and 20 atopic dogs. Both groups mounted an IgG response to multiple antigens from the mite. There was no significant difference in the number of bands recognised, or the molecular weights of the bands, between the two groups. The two most obvious bands in both groups were proteins with molecular weights of 98Kd (likely to be the high molecular weight allergen Der f 15) and 44 Kd, although dogs in both groups recognised a similar pattern of other antigens. The magnitude of the IgG response was greater in the atopic group although this was not statistically significant. The results indicate that the immune system of both healthy and atopic dogs generates an IgG response to multiple antigens from *Dermatophagoides farinae*. As some of these antigens (such as the 98Kd and 44Kd proteins) are also targeted by IgE in atopic dogs, immunoglobulin class switching in response to Th2 cytokines may not be as dominant a process as has been proposed.

2. Hou C, Day MJ, Nuttall TJ, and Hill PB. (2006) Evaluation of IgG subclass responses against *Dermatophagoides fairnae* allergens in healthy and atopic dogs. Veterinary Dermatology. 17: 103-110.

A semi-quantitative chemiluminescent western blot analysis system was developed and validated to evaluate allergen-specific IgG subclass responses to electrophoretically separated allergens of *Dermatophagoides farinae* in 20 healthy and 20 atopic dogs.
Both groups mounted IgG1 and IgG4 responses to multiple antigens from the mite while *D. farinae*-specific IgG2 and IgG3 levels were undetectable using standard blotting conditions. The profiles of the IgG1 and IgG4 responses were similar in the two groups, both in terms of the number of bands recognised, and their molecular weights. The most commonly recognised bands in both healthy and atopic groups

were 98 kDa and 18 kDa antigens for IgG1 and 98 kDa and 45 kDa antigens for IgG4. The number of bands recognised per dog did not differ significantly between the two groups. The overall *D. farinae*-specific IgG1 and IgG4 responses were slightly higher in the healthy group but were not significantly different. The IgG1 responses to the 98 kDa and 18 kDa antigens and the IgG4 responses to the 98 kDa and 45 kDa were slightly higher in the healthy group but again the differences were not statistically significant. These results suggest that different allergens produced by *Dermatophagoides farinae* can induce different subclass responses. However, as these responses are seen in both healthy and atopic dogs, they are likely to merely represent recognition of foreign proteins that are presented to the immune system, rather than being involved in the pathogenesis of atopic dermatitis.

3. Hou C, Nuttall TJ, Day MJ, and Hill PB. (2005) *Dermatophagoides farinae*-specific IgG subclass responses in atopic dogs undergoing allergen-specific immunotherapy. In: Hillier A, Foster AP, and Kwochka KW eds. Advances in Veterinary Dermatology, Vol 5. Oxford: Blackwell Publishing. 70-81.

The molecular and immunologic mechanisms involved with successful allergen-specific immunotherapy (ASIT) have not been completely elucidated. The aim of this study was to characterize the changes in *Dermatophagoides farinae*-specific total IgG and IgG subclasses during ASIT of dogs with atopic dermatitis. Twenty-one dogs with *D. farinae* hypersensitivity were treated with alum-precipitated vaccines for 9 months. Serum samples were collected before and after 3, 6, and 9 months of therapy and used to probe western blots containing separated proteins of *D. farinae*. IgG responses were detected using a polyclonal antibody and a colourimetric substrate whereas IgG subclasses were detected using a panel of monoclonal anti-IgG antibodies and chemiluminescence. The blots were analysed using a semi-quantitative digital image analysis system that provided the number and molecular weight of bands, as well as their intensity, which was related to IgG concentration. Prior to ASIT, all dogs showed an IgG, IgG1 and IgG4 response to multiple proteins of different molecular weights, the most common being 98Kd and 44Kd. There was virtually no detectable IgG2 or IgG3 response. During ASIT, the

total IgG, IgG1 and IgG4 response to *D. farinae* antigens varied widely between dogs and could increase, decrease, fluctuate, or remain the same, but there was no induction of IgG2 or IgG3 antibodies. There were no significant increases in total IgG or IgG subclass responses in dogs showing a complete or partial response to ASIT. However, dogs showing no response to ASIT had significantly higher *D. farinae*-specific total IgG levels prior to the start of therapy, compared to dogs that responded to ASIT .

4. Hou C, Griffin CE, and Hill PB. (2007) Changes of *Dermatophagoides farinae*-specific immunoglobulin G in atopic dogs undergoing allergen-specific immunotherapy using aqueous vaccines. Submitted to Veterinary Dermatology.

The molecular and immunologic mechanisms involved with successful allergen-specific immunotherapy (ASIT) have not been completely elucidated. The aim of this study was to characterize the changes in *Dermatophagoides farinae* (*D. farinae*)-specific total IgG in atopic dogs undergoing ASIT using aqueous vaccines. Fifteen dogs with *D. farinae* hypersensitivity were treated with aqueous vaccines for a minimal of 2 months. Serum samples were collected before and after therapy and used to probe western blots containing separated proteins of *D. farinae*. IgG responses were detected using a polyclonal goat anti-canine IgG antibody and a chromogenic substrate 3,3'-diaminobenzidine (DAB). The blots were analysed using a semi-quantitative digital image analysis system that provided the number and molecular weight of bands, as well as their intensity, which was related to IgG concentration. Prior to and during ASIT, all dogs showed an allergen-specific IgG responses to various antigens of the house dust mite *D. farinae*. During ASIT with aqueous vaccines, there was a significant augmentation in the quantity of *D. farinae*-specific IgG antibodies to various antigens from the mite (p=0.015). The significant increase in the IgG response to the 98-kDa band (p=0.015), the 50-70 kDa bands (p=0.012) and the 30-45 kDa bands (p=0.035). This finding provides some support for the hypothesis that ASIT induces blocking antibodies because of possible competition of IgG with IgE for antigen binding sites on effector cells.

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IgG responses to antigens from *Dermatophagoides farinae* in healthy and atopic dogs

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Abstract

In this study, we used a semi-quantitative electrophoresis and immunoblotting technique to characterise the IgG response to antigens from *Dermatophagoides farinae* in 20 healthy and 20 atopic dogs. Both groups mounted an IgG response to multiple antigens from the mite. There was no significant difference in the number of bands recognised, or the molecular weights of the bands, between the two groups. The two most obvious bands in both groups were proteins with molecular weights of 98 kDa (likely to be the high molecular weight allergen Der f 15) and 44 kDa, although dogs in both groups recognised a similar pattern of other antigens. The magnitude of the IgG response was greater in the atopic group although this was not statistically significant. The results indicate that the immune system of both healthy and atopic dogs generates an IgG response to multiple antigens from *D. farinae*. As some of these antigens (such as the 98 and 44 kDa proteins) are also targeted by IgE in atopic dogs, immunoglobulin class switching in response to Th2 cytokines may not be as dominant a process as has been proposed.
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Keywords: Atopic dermatitis; *Dermatophagoides farinae*; IgG; Dogs

1. Introduction

Atopic dermatitis (AD) is one of the most common pruritic skin diseases in the canine population and is characterised by an imbalance in the response of the immune system upon allergen-recognition (Hill and DeBoer, 2001; Hill and Olivry, 2001). Since Wittch (1941) first described a dog suffering from AD, much research has been performed to elucidate the pathogenesis of the disease, yet the exact mechanisms are still unknown. One key aspect of the pathogenesis is the production of allergen-specific IgE in response to innocuous environmental allergens (Halliwell and DeBoer, 2001). This is thought to result from initial polarisation of the Th2 cytokine profile leading to increased production of interleukin 4 and immunoglobulin class switching (Olivry et al., 1999; Nuttall et al., 2002). Recent studies have shown that a high

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molecular weight chitinase derived from the house dust mite *Dermatophagoides farinae* (*D. farinae*), termed Der f 15, is the most important allergen in dogs, whereas the low molecular weight group I proteases appear to be less involved (Noli et al., 1996; McCall et al., 2001; Nuttall et al., 2001).

The role of IgG in canine atopic dermatitis is less well understood. Hill et al. (1995) showed that total IgG concentrations were significantly higher in atopic dogs compared to healthy controls. Willemse et al. (1985) also demonstrated that IgGd was capable of inducing an anaphylactic reaction and suggested that IgGd might be important in the pathogenesis of canine atopic dermatitis. However, Lian and Halliwell (1998) showed that significantly higher levels of *Dermatophagoides pteronyssinus*-specific IgGd were present in normal dogs compared to atopic dogs, which argues for a protective role for IgGd. In contrast, Fraser et al. (2004) have recently shown that concentrations of total IgG1 are higher in atopic dogs than in healthy dogs, suggesting that the atopic state also leads to activation of IgG subclass production in addition to IgE. A role for IgG has also been proposed in the mechanism of action of allergen-specific immunotherapy (ASIT). Hites et al. (1989) detected IgG antibodies with specificity for various allergens in the majority of non-atopic individuals and in all atopic subjects, but concentrations were highest in atopic dogs receiving allergen-specific immunotherapy. Furthermore, Fraser et al. (2004) have shown that concentrations of total IgG1 can increase following allergen-specific immunotherapy.

To date, compared to IgE, there is limited information on the specific IgG response to *D. farinae* antigens in atopic dogs. The aim of this study, therefore, was to develop and validate a semi-quantitative blot analysis system and use it to characterise and quantify the IgG response using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

2. Materials and methods

2.1. Serum samples

Client-owned dogs suffering from atopic dermatitis ($n = 20$) were recruited from the Dermatology Clinic at the University of Edinburgh Hospital for Small Animals. The diagnosis of atopic dermatitis was based on a combination of a compatible history and clinical signs (Willemse, 1986), exclusion of other pruritic skin diseases (e.g., ectoparasite infestation and adverse food reaction), and the presence of at least one positive reaction either in an intradermal test or a commercially available allergen-specific IgE assay (Heska Corporation, CO, US). Coat brushings, skin scrapings and trial therapy were used to rule out ectoparasites. A 6-week home-cooked diet trial was conducted to eliminate the possibility of adverse food reactions. Seborrhoea, staphylococcal pyoderma and *Malassezia* infections that occurred secondarily to atopic dermatitis were managed appropriately. No anti-inflammatory medication was given for at least 3 weeks prior to intradermal skin testing. All serum samples were collected prior to the initiation of allergen-specific immunotherapy.

Control samples were taken from healthy dogs ($n = 20$), either belonging to clients or staff members, or dogs presented from a local rescue centre. None of the healthy dogs had a history or clinical signs of pruritic skin disease or systemic conditions likely to affect immune function. All procedures were performed with UK Home Office approval.

2.2. D. farinae extracts

A freeze-dried crude extract from *D. farinae* (Greer Laboratories, Lenoir, USA) was reconstituted in sterile phosphate-buffered saline (PBS) (pH 7.4) to a final concentration of 1 mg/ml and 200 μl aliquots were stored at −70 °C.

2.3. SDS-PAGE and protein transfer

SDS-PAGE was performed according to Laemmli's method (Laemmli, 1970) using a 10% Tris–glycine polyacrylamide resolving gel and a 4% stacking gel in a discontinuous buffer system containing 25 mM Tris–HCl, 0.2 M glycine and 0.1% SDS, pH 8.3. *D. farinae* extract (50 μg) and molecular weight standards were diluted 1:1 and 1:20, respectively, with reducing sample buffer (125 mM Tris–HCl, 4% SDS, 20% glycerol, 0.005% bromophenol blue and 10% 2-mercaptoethanol) and heated at 95 °C for 5 min. The extract was then loaded into a single broad well with

the molecular weight marker alongside and the electrophoresis was run at 200 V for 40 min. Separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Immobilon™-P Transfer Membrane, Millipore Corporation, Bedford, MA, USA) in a Bio-Rad Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell using 0.3 M Tris–HCl/10% methanol (pH 10.4) on the lower anode, 25 mM Tris–HCl/10% methanol (pH 10.4) on the upper anode and 25 mM Tris–HCl/40 mM 6-amino-*n*-hexonic acid/10% methanol (pH 9.4) on the cathode according to the manufacturers instructions. The transfer was run at 80 mA per minigel for 1 h. Membranes were then dried and stored at 4 °C.

2.4. Immunoblotting

The molecular weight markers (Bio-Rad Laboratories Ltd., Hertfordshire, UK) and a strip to identify protein bands were removed from the edge of the membrane and stained with 0.25% Coomassie Brilliant Blue R-250 (BDH, Dorset, UK). The remainder of the membrane was then cut longitudinally into 4-mm strips and placed into individual lanes of an incubation tray, blocked with 5% skimmed milk/Tris-buffered saline (TBS)/0.1% Tween-20 (TTBS) for 1 h at room temperature and washed with washing buffer (TTBS) three times for 5 min. Preliminary experiments were performed to determine the optimal dilution of sera and the primary antibody to yield strong bands against a clear background (data not shown). Serum samples were diluted to 1:100 in dilution buffer (1% skimmed milk/TTBS) and 1 ml was added to each lane and incubated for 1 h at room temperature. After washing as described above, the strips were incubated with 1 ml of 1:1000 horseradish peroxidase-conjugated goat anti-dog IgG (Bethyl Laboratories Inc., Montgomery, TX, USA) diluted in dilution buffer for another hour at room temperature, and washed thoroughly with washing buffer. One millilitre of 3,3′-diaminobenzidine (DAB; Vector Laboratories Inc., Burlingame, CA, USA), prepared according to the manufacturers recommendations, was finally added to each strip for 1 min and then removed by washing in distilled water. The strips were air-dried overnight prior to analysis.

The specificity of the binding of the anti-canine IgG to dog IgG and not *D. farinae* proteins was confirmed by performing experiments in which serum samples were omitted. No bands were visible in these experiments (data not shown). The specificity of the anti-canine IgG is verified by the manufacturer, but any potential cross-reactivity of the reagent with canine IgE would not be relevant in these experiments because serum concentrations of allergen-specific IgE are so low that they cannot be detected using colorimetric blots, even when employing specific anti-canine IgE reagents (Chen et al., 2002b).

2.5. Digital image analysis

The strips, along with the molecular weight standard, were aligned and digitally scanned using a flatbed scanner (Epson 1650, Hemel, Hempstead, UK) set at 8-bit grey scale. The bands were analysed by importing the scanned images into an image analysis software (Kodak Digital Science™ 1D Image Analysis Software, Kodak, Rochester, NY, USA). Band sensitivity was set at 0 and the net intensity of individual bands was calculated automatically in pixels. This system allowed measurement of both band molecular weight and density without operator bias. Bands with molecular weights >200 or <10 kDa were not analysed because they were outwith the molecular weight marker range. Numerical data was exported to MINITAB™ statistical software (Minitab Inc., PA, USA) for analysis.

2.6. Validation of semi-quantitative analysis system

In order to validate the immunoblotting and image analysis system for semi-quantitative measurements, experiments were performed to determine linearity, sensitivity, saturation effects and repeatability. To confirm that changes in serum concentration resulted in a linear standard curve, blots containing separated *D. farinae* proteins were probed with 3-fold serial dilutions of a standard serum sample from 1:10 to $1{:}10^6$. The standard serum sample was chosen because it gave a positive response to a number of protein bands in preliminary experiments. Fig. 1 shows representative curves for a high molecular weight band (98 kDa) and a lower molecular weight band (44 kDa). The intensity of each band changed in a linear fashion between log serum dilutions of 1:10 and 1:1000, which represented the lower limit of

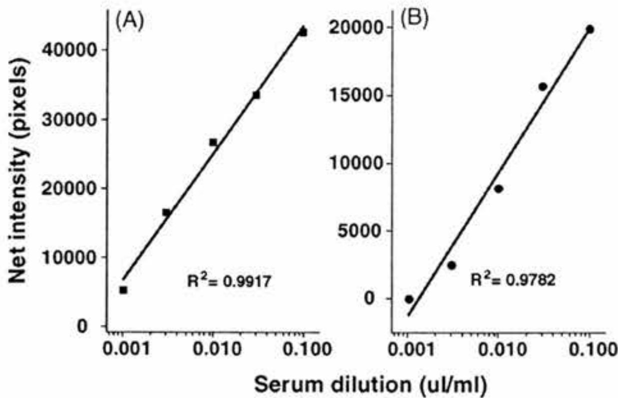

Fig. 1. Effect of serum dilution on net intensity of bands. Serum was diluted 3-fold between 1:10 and 1:10^6 and used to probe blots containing separated *D. farinae* proteins. (A) Curve for a 98 kDa band; (B) curve for a 44 kDa band. Linearity was obtained over the whole molecular weight range with serum dilutions ranging between 1:10 and 1:1000, the lower limit of sensitivity.

sensitivity. Hence, by using serum dilutions of 1:100, the system could be used to reliably semiquantify IgG concentrations over a range that was 10× higher or 10× lower than those normally found in serum.

To ensure that band intensity was not affected by colorimetric saturation, identical strips containing separated *D. farinae* proteins probed with canine sera were incubated with the DAB substrate for different periods of time ranging between 10 s and 20 min. Linear development of colour intensity occurred between 10 s and 5 min, after which a plateau was reached (data not shown). Hence, routine development of the blots for 1 min did not affect the semi-quantitative assessment of IgG responses.

To determine the repeatability of the system, 15 identical strips, all probed with the same serum sample at a dilution of 1:100, were assayed as described above. The coefficients of variation of net intensity for bands over the whole molecular weight range was typically around 10%, with a maximum of 15%. These were considered satisfactory for semi-quantitative analysis.

2.7. Statistical analysis

The Mann–Whitney test was used to compare healthy and atopic dogs for the number of bands recognised, the molecular weight range of the bands observed, the total intensity of the bands in each group and the intensity of the most important individual bands. A *p*-value of <0.05 was considered to be significant.

3. Results

A representative set of IgG immunoblots of individual dog sera from the two groups is shown in Fig. 2. Both healthy and atopic dogs mounted an IgG response to multiple antigens from *D. farinae*. The two most visually obvious bands in both groups were seen at molecular weights of approximately 98 and 44 kDa (Fig. 2). The number of bands recognised did not differ significantly between the two groups ($p = 0.6652$; Fig. 3) and varied between 3 and 12 in the healthy group and 2 and 10 in the atopic group, with median band numbers of 6.5 and 6, respectively.

The percentage of dogs recognising antigens of various molecular weights is shown in Fig. 4. In both the healthy and atopic groups, two peaks of frequency were seen. The first occurred at around 100 kDa and predominantly comprised responses to the 98 kDa antigen. The second occurred between 30 and 60 kDa, a major proportion of which represented responses to the 44 kDa antigen. However, IgG responses were seen to multiple other bands ranging in molecular weights from 20 to 180 kDa (Figs. 2 and 4).

The intensity of each band, and therefore, the magnitude of the IgG response to that particular protein(s), was provided by the digital analysis software. The intensity of response in each dog to proteins of various molecular weights is shown in Fig. 5. The most intense bands were seen at molecular weights clustered around 100 and 44 kDa. Hence, not only were these proteins the most commonly recognised (as seen in Fig. 4), they also resulted in the greatest IgG response. The average of the total band intensity in each strip (i.e., for each dog) was 8335 in the healthy group and 9997 in the atopic group; this difference approached statistical significance ($p = 0.0697$). The total response to the 98 kDa protein was similar in the atopic group (net intensity 18411) and healthy groups (net intensity 16953) and was not significantly different ($p = 0.4094$). However, the total response to the 44 kDa antigen in the atopic group (net intensity 11088) was almost double that seen in the healthy group (net intensity 6410). Based

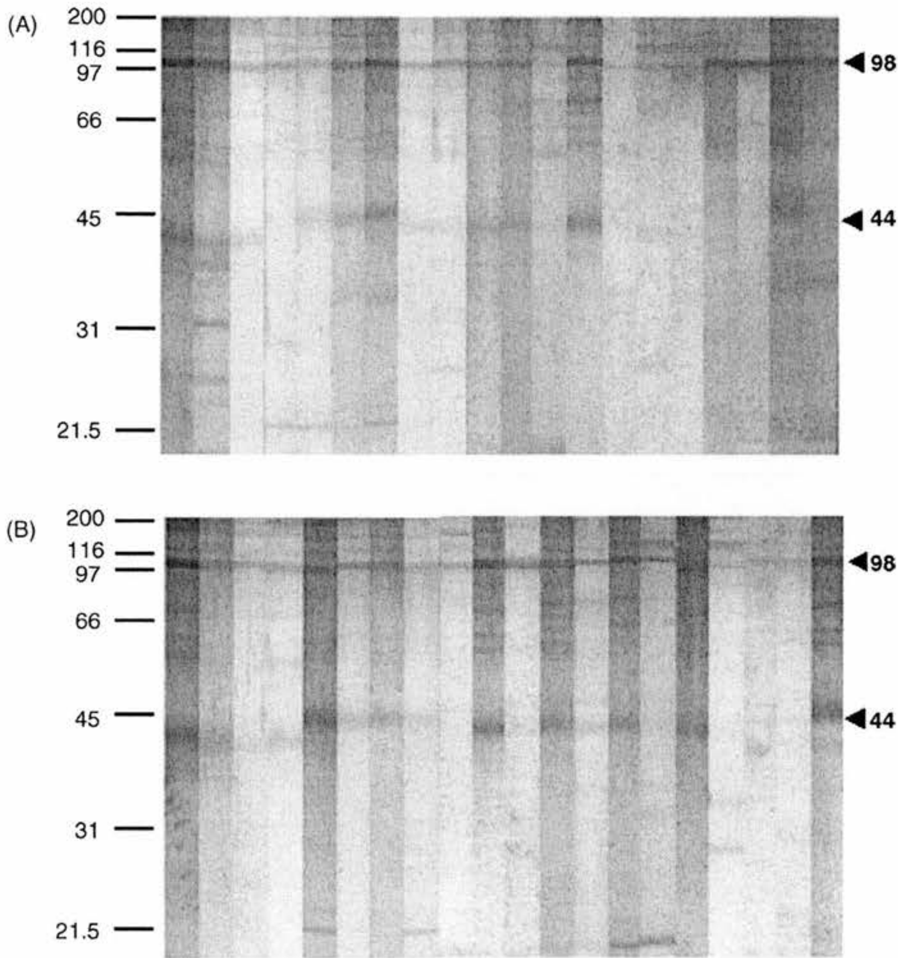

Fig. 2. Binding of canine IgG to separated proteins of *D. farinae* in 20 healthy dogs (A) and 20 atopic dogs (B). Each strip represents one dog.

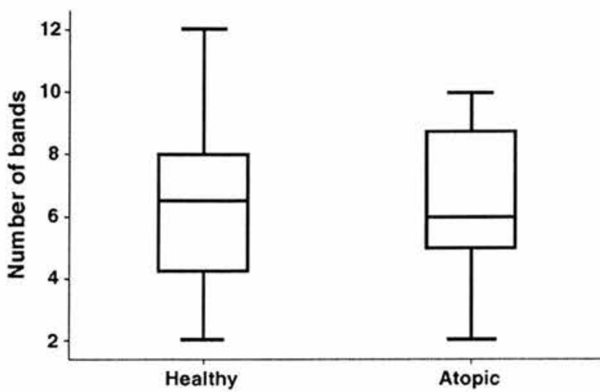

Fig. 3. Number of *D. farinae* bands recognised by canine IgG in healthy and atopic dogs. Each box and whisker plot summarises the response of 20 dogs. The box represents the 25th and 75th quartiles with the horizontal bar indicating the median. The whiskers indicate the range.

on the standard curve for this protein, this actually represented a 6-fold increase in serum concentration, but the difference between groups was again not statistically significant ($p = 0.2689$).

4. Discussion

In this study, we compared the *D. farinae*-specific IgG responses in healthy dogs and dogs with atopic dermatitis. The profile of IgG binding was similar in the two groups, both in terms of the number of bands recognised and their molecular weights. The percentage of individual dogs recognising these proteins was also similar in the two groups. These results indicate that *D. farinae* produces a number of proteins that are

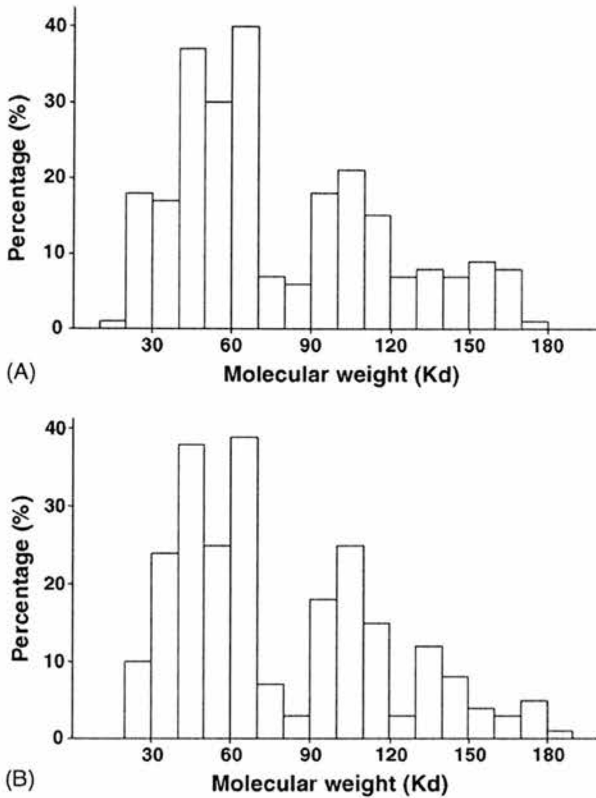

Fig. 4. Proportion of healthy (A) and atopic (B) dogs recognising *D. farinae* allergens. Antigens are grouped in 10 kDa intervals and ranged from 15 to 185 kDa. A similar pattern was detected in both groups.

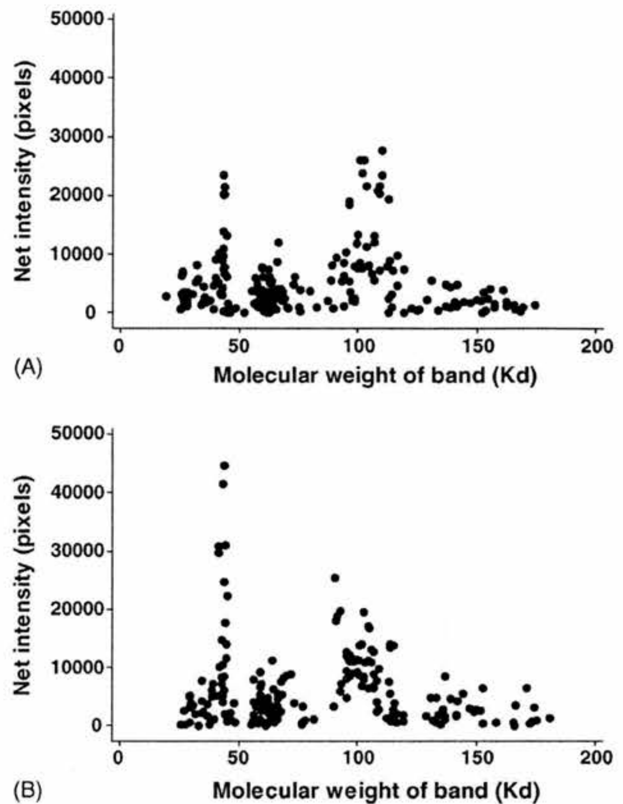

Fig. 5. Plot of band molecular weight against band net intensity in healthy (A) and atopic (B) dogs. Each dot represents an IgG response to a *D. farinae* protein in an individual dog. Note the two peaks of intensity around 100 and 44 kDa.

recognised by the canine immune system, both in healthy and atopic dogs.

The strength of the IgG response appeared to be greater overall, and for the 44 kDa band, in the atopic dogs than in the healthy controls. The lack of significant differences between the groups might imply that this difference was biologically irrelevant but this must be interpreted with caution. A larger number of dogs in each group would have increased the statistical power, which might have yielded a significant difference.

These results are in accordance with previous findings that demonstrated the presence of antigen-specific IgG antibodies in both healthy and atopic dogs using ELISAs (Willemse et al., 1985; Hites et al., 1989; Day et al., 1996; Lian and Halliwell, 1998). Furthermore, the increased antigen-specific IgG response in the atopic group would be consistent with previous reports that showed higher concentrations of total IgG (Hill et al., 1995) and total IgG1 (Fraser et al., 2004) in atopic dogs. However, it must be born in mind that these two previous studies measured total, and not antigen-specific, antibody concentrations. The total IgG responses seen in these studies could be directed at antigens other than *D. farinae* such as additional environmental allergens or proteins produced by *Malassezia* or staphylococcal organisms. Secondary infection with these pathogens is common in dogs with atopic dermatitis and previous studies have shown significantly greater IgG responses in atopic dogs to both *Malassezia* antigens (Nuttall and Halliwell, 2001; Chen et al., 2002a) and staphylococcal proteins (Halliwell, 1987; Morales et al., 1994; Shearer and Day, 1997).

The most important *D. farinae* antigens recognised, both in terms of frequency and magnitude, were 98 and 44 kDa proteins. We, and others, have shown previously that bands with the same molecular weights

are recognised in IgE blots (Noli et al., 1996; McCall et al., 2001; Nuttall et al., 2001), although the 44 kDa protein is only seen when reducing buffers are used (Nuttall et al., 2001), suggesting that it might be a part of a bigger antigen. It is likely that the 98 kDa protein is the same as the high molecular weight chitinase that has recently been designated Der f 15 (McCall et al., 2001). This protein is the most important allergen in atopic dogs that is recognised by IgE. However, the substantial IgG response to this protein indicates that distinct immune responses dominated by either isotype do not occur. Hence, although IgE responses are a critical component in the pathogenesis of canine atopic dermatitis, the concept of Th2 cytokine controlled class switching is clearly not an absolute phenomenon.

Taken together, the results of this and previous studies suggest that dogs mount an IgG response to multiple environmental antigens, including house dust mites. This response is similar in healthy and atopic dogs, but is possibly greater in magnitude in atopic dogs. Hence, the presence of *D. farinae* antigens in the environment does not appear to be ignored by the canine immune system in healthy dogs and yet the IgG response does not appear to be protective against the development of clinical atopic disease. Whether the IgG response plays any role in the pathogenesis of the disease remains to be determined.

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Evaluation of IgG subclass responses against *Dermatophagoides farinae* allergens in healthy and atopic dogs

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Abstract

A semiquantitative chemiluminescent Western blot analysis system was developed and validated to evaluate antigen-specific IgG subclass responses to electrophoretically separated proteins of *Dermatophagoides farinae* in healthy and atopic dogs.

Both groups mounted similar *D. farinae*-specific IgG1 and IgG4 responses to multiple antigens, but IgG2 and IgG3 responses were difficult to detect. The most commonly recognized bands in both groups were 18 and 98 kDa antigens for IgG1 and 18, 45, 66, 98, 130 and 180 kDa for IgG4. The number of bands recognized per dog did not differ significantly, but significantly more atopic dogs had an IgG1 response to a 180 kDa protein. The overall *D. farinae*-specific IgG1 and IgG4 responses were slightly higher, but not significantly different, in the healthy group. The results suggest that some antigens produced by *D. farinae* can induce different subclass responses. However, as most of these responses are seen in both healthy and atopic dogs, they are likely to merely represent recognition of foreign proteins presented to the immune system, rather than involvement in the pathogenesis of atopic dermatitis. The role of the 180 kDa antigen warrants further study.

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Introduction

Atopic dermatitis (AD) is a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features.[1] Affected individuals mount an aberrant immune response against innocuous environmental allergens, most commonly to house dust mite antigens from *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*.[2–8] Much research has been performed to elucidate the mechanisms underlying AD in dogs, especially in relation to the role of IgE which plays an important role in the development of immediate hypersensitivity. It has been shown that serum allergen-specific IgE antibodies are usually elevated both in humans[9,10] and in dogs[11,12] with atopic diseases. However, the role of IgG remains controversial and paradoxical. In humans, IgG concentrations are elevated in various allergic conditions.[13–15] Both total serum IgG[16] or allergen-specific IgG can be raised in dogs suffering from AD, although dogs with no apparent clinical sensitivity to a particular allergen can also have elevated allergen-specific IgG.[17–19]

IgG is not a homogenous molecule. In humans, there are four IgG subclasses, distinguished by differences in γ-chain sequence and electrophoretic mobility. They are numbered according to their decreasing mean serum concentrations as IgG1, IgG2, IgG3 and IgG4. In dogs, four IgG subclasses have also been identified[20] and similarly named: IgG1 (mean serum concentration 8.17 mg mL^{-1}), IgG2 (8.15 mg mL^{-1}), IgG4 (0.95 mg mL^{-1}) and IgG3 (0.36 mg mL^{-1}). Non-IgE anaphylactic antibodies have been identified as IgG4 in man[21] and guinea pigs,[22] IgG1 in mice,[23] IgG2a in rats[24] and IgG2 in sheep.[25] Studies by Day[17] suggested that IgG1 and IgG4 were involved in canine AD, depending on the allergen. Using a panel of monoclonal antibodies (mAbs) directed against the four subclasses of canine IgG,[20,26] it was shown that the IgG response to *D. farinae* was dominated by the IgG4 subclass.[17] However, the specific proteins involved could not be identified in the ELISAs used.

The situation in dogs is further complicated by the description of IgGd,[27] a subclass that does not correspond with the 1–4 nomenclature.[17] Willemse *et al.*[19,27] demonstrated the presence of non-IgE anaphylactic antibodies, termed IgGd, in the sera of atopic dogs and suggested that this subclass could initiate immediate hypersensitivity. However, its role in atopic diseases is uncertain; IgGd directed against apparently irrelevant allergens is present in atopic dogs, and there are high levels of IgGd in normal dogs to the common allergens *D. pteronyssinus* and *D. farinae*.[12] It is possible that IgGd corresponds to IgG1 (R. Halliwell, personal communication), but this has not been proved. In contrast, antiserum specific for IgGd failed to identify any of the four canine IgG subclasses in purified form in ELISAs.[17] This may reflect the fact that IgGd is a myeloma protein, and the antiserum may not necessarily recognize native protein.

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Nothing is known about the individual allergenic proteins targeted by IgG subclasses in dogs with AD because these have not been evaluated by ELISAs and the crude allergen extracts used previously. Therefore, the aim of this study was to develop and validate a semiquantitative, digital, chemiluminescent Western blot analysis system to evaluate the allergen-specific IgG subclass response to electrophoretically separated allergens of *D. farinae* in dogs with atopic dermatitis.

Materials and methods

Dogs and serum samples

Dogs suffering from AD (n = 23) were recruited from the dermatology clinic at the Royal (Dick) School of Veterinary Studies, Edinburgh. The diagnosis of AD was based on a combination of compatible history and clinical signs,[28] exclusion of other pruritic skin diseases (e.g. ectoparasite infestation and adverse food reaction), and the presence of at least one positive reaction (including *D. farinae*) in either an intradermal test or a commercially available allergen-specific IgE assay (Allercept®, Heska Corporation, Fort Collins, CO, US). Coat brushings, skin scrapings and trial therapy were used to rule out ectoparasites and a 6-week home-cooked diet trial was conducted to eliminate the possibility of adverse food reactions. Concurrent seborrhoea, staphylococcal pyoderma and *Malassezia* infections were managed appropriately with systemic and topical medications. No glucocorticoids or antihistamines were given for at least 3 weeks prior to intradermal testing. All serum samples were collected prior to the initiation of allergen-specific immunotherapy.

Control samples were taken from healthy dogs (n = 20) presented for euthanasia from a local rescue centre with no history or clinical signs of skin disease or conditions likely to alter immune function.

Blood samples were collected from atopic dogs (n = 23) by jugular venepuncture and from healthy dogs by cardiac puncture immediately after euthanasia. Serum was separated by centrifugation and stored at −20 °C.

Dermatophagoides farinae extract

Lyophilized whole-body *D. farinae* crude extract (Greer Laboratories, Lenoir, NC, USA) was reconstituted to 1 mg mL^{-1} in sterile phosphate-buffered saline (PBS, pH 7.5) and 200 µL aliquots were stored at −20 °C.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli[29] using a 10% Tris-glycine polyacrylamide (ProtoGel®, National Diagnostics, UK) separating gel and 4% stacking gel in a discontinuous buffer system containing 25 mmol L^{-1} Tris-HCl (Trizma® hydrochloride; Sigma-Aldrich, Dorset, UK), 200 mmol L^{-1} glycine (Fisher Scientific, Loughborough, UK) and 0.1% SDS (Fisher Scientific, UK), pH 8.3. 100 µL of *D. farinae* extract and molecular weight markers were diluted 1 : 1 and 1 : 20, respectively, with reducing sample buffer containing125 mmol L^{-1} Tris-HCl, 4% SDS, 20% glycerol (BDH Chemicals, Poole, UK), 0.005% bromophenol blue (BDH, UK) and 10% 2-mercaptoethanol (Sigma-Aldrich, UK) and then heated at 95 °C in a water bath for 5 min. The extract was then loaded into one broad well across the top of the gel and the electrophoresis run at 200 V for 40 min (Mini-Protean II®, Biorad, Hercules, USA).

Western blots

Separated *D. farinae* proteins were transferred to polyvinylidene fluoride (PVDF) microporous membranes (Millipore Immobilon™-P Transfer Membrane, Millipore Corp., Bedford, MA, USA) in a semidry electrophoretic transfer cell (Trans-Blot® SD, Bio-Rad, Atlanta, GA, USA) using 300 mmol L^{-1} Tris-HCl/10% methanol (pH 10.4) on the lower anode, 25 mmol L^{-1} Tris-HCl/10% methanol (pH 10.4) on the upper anode and 25 mmol L^{-1} Tris-HCl/40 mmol L^{-1} 6-amino-n-hexonic acid/10% methanol (pH 9.4) on the cathode. The transfer was run at 80 mA per minigel for 1 h. Membranes were dried and stored at 4 °C. Transfer quality was checked by staining gels and molecular weight standards blotted onto the membrane with 0.25% Coomassie Brilliant Blue R-250 (BDH Chemicals, UK).

Monoclonal antibodies

A panel of four mouse monoclonal antibodies specific for the subclasses of canine IgG were used:[20,26,30,31] mAb B6 with restricted specificity for canine IgG1 and IgG3, but specifically reactive with IgG1 in serological and immunohistochemical assays; mAb E5 specific for canine IgG2; mAb A3G4 specific for canine IgG3; mAb A5 with restricted specificity for canine IgG2 and IgG4 but reactive specifically with IgG4 in serological and immunohistochemical assays. The specific activity of the subclass-restricted mAbs B6 and A5 is probably due to conformational differences between the target IgG subclasses in solid-phase assay vs. other serological and immunohistochemical techniques.[30,31] The mAbs A5, E5 and B6 were used as 45% ammonium sulphate precipitates of tissue culture supernatants, and A3G4 was used as undiluted tissue culture supernatant as it was in lower supply.

Assessment of the mAbs used in immunoblotting

To determine if the monoclonal antibodies against IgG subclasses would work in immunoblotting, a commercial preparation of canine IgG purified by ion-exchange chromatography (Sigma-Aldrich, Dorset, UK) was used. One hundred micrograms purified canine IgG diluted in both reducing buffer and nonreducing buffer was separated by SDS-PAGE, transferred to a PVDF membrane and cut into strips. After blocking and thorough washing, each mAb was added to individual strips at a dilution of 1/200 in buffer and incubated overnight at room temperature (RT). After further washing, bovine antimouse IgG conjugated with horseradish peroxidase (Bethyl Laboratories, Montgomery, TX, USA) at 1/1000 dilution was added to each strip before incubation at RT for 2 h. The luminol substrate ECL (ECL, Amersham-Pharmacia Biotech, Little Chalfont, UK) was used to develop the chemiluminescent signals, and the bands were visualized using an image acquisition system as described below. Preliminary experiments showed that strong signals could be obtained for IgG1 and IgG4, but not for IgG2 or IgG3. IgG2 and IgG3 responses could only be detected after prolonged exposures (2 h) to increase the sensitivity (compared to 32 min for IgG1 and 10.6 min for IgG4).

Immunoblotting of serum samples against Dermatophagoides farinae proteins

Blotting membranes with transferred separated *D. farinae* proteins were cut longitudinally into 4-mm wide strips to allow probing with serum samples from different dogs. Strips were inserted into individual lanes of an eight-channel incubation tray (Bio-Rad, UK), blocked with 5% skimmed milk (Marvel, Sainsbury, UK) in Tris-buffered saline (TBS, pH 7.5) containing 0.02 mol L^{-1} Tris-HCl and 0.5 mol L^{-1} NaCl (Sigma-Aldrich, UK) for 1 h at RT, and washed with 0.1% Tween 20 (Fisher Scientific, UK)/TBS (TBS/T) three times for 5 min. For effective immunoblotting, it was important that each mAb was used at a dilution that gave a high signal:noise ratio. After a series of titrations, the optimum dilutions were found to be 1/20 for serum samples, 1/200 for the mABs, and 1/1000 for bovine antimouse IgG. All reagents were diluted in dilution buffer (1% skimmed milk/TBS/T).

After blocking, strips were probed with individual dog sera (1/20 dilution) for 1 h at RT, followed by a 1-h incubation with one of the four mAbs (1/200) with specificity for each canine IgG subclass. Horseradish peroxidase-conjugated bovine antimouse IgG (Bethyl Laboratories, USA) at 1/1000 was added for another hour. Incubation steps were accompanied by gentle agitation, with frequent changes of washing buffer between each step. The protein surface of each strip was then covered with the luminol solution (ECL, Amersham-Pharmacia Biotech, Little Chalfont, UK) for 1 min and visualized on an image station as described below.

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Digital image acquisition and analysis

The chemiluminescent substrate ECL was used for blot visualization because of its higher sensitivity than colourimetric substrates. Higher sensitivity was required because the concentrations of IgG subclasses are obviously lower than total IgG,[30] and the concentrations of allergen-specific IgG subclasses lower still. After draining off excess liquid, the strips were wrapped in cling film and placed protein side down on a Kodak Digital Science™ Image Station 440CF (Kodak, Rochester, NY, USA) along with Coomassie blue-stained molecular weight standards. Images were obtained with a charge coupled device (CCD) camera using multiple exposures to eliminate the possibility of image saturation (see below). Kodak Digital Science™ 1D Image Analysis software was used to detect the bands on the blots and to determine the signal magnitude of individual bands and their molecular weights by analysing their positions relative to the molecular weight standards. This system allowed measurements of both band molecular weight and density without operator bias. Data were then exported to MINITAB™ statistical software (Minitab Inc., PA, USA) for analysis.

Validation of the semiquantitative, chemiluminescent analysis system

To validate the chemiluminescent immunoblotting and image analysis system for semiquantitative measurements, experiments were carried out to determine linearity, sensitivity, saturation effects and repeatability. To confirm that changes in serum concentration resulted in a linear standard curve, blots containing separated *D. farinae* proteins were probed with threefold serial dilutions of a reference serum sample (from a healthy dog which gave a positive signal in preliminary experiments) from 1 : 10 to 1 : 10^5 in duplicate. To ensure that band intensity was not affected by saturation, strips containing separated *D. farinae* proteins probed with canine sera and antibodies were imaged using different exposure and capture times. To determine system repeatability, 10 identical strips all probed with the same serum sample were assayed as described above and imaged simultaneously.

Statistical analysis

The Mann–Whitney test was used to compare healthy and atopic dogs for the number of bands recognized, the total intensity of the bands in each group and the intensity of the most important individual bands. The frequency with which a particular band was recognized within the two groups was compared by Fisher's exact test. A *P*-value of < 0.05 was considered to be significant.

Results

Validation of the semiquantitative, chemiluminescent analysis system

Using the standard dilutions, incubation times and exposure times, no signals were seen with IgG2 or IgG3. As in the preliminary experiments using whole IgG, *D. farinae*-specific IgG2 and IgG3 could only be detected when assay sensitivity was increased dramatically by using low dilutions of serum and mAbs, and prolonged incubation and exposure times. Even then, the bands were very weak. As the IgG2 and IgG3 responses were so low, it was not possible to generate standard curves for semiquantitative analysis. For IgG1 and IgG4, linear standard curves were obtained using serial serum dilutions. Figure 1 shows curves generated using a reference serum sample from a healthy dog. In Fig. 1A, linear standard curves can be seen for two proteins recognized by IgG1 with molecular weights of 98 and 18 Kd. In Fig. 1B, linear standard curves can be seen for the IgG4 response. The linearity of each curve extended over a range of serum dilutions from 1 : 10 to 1 : 1000, which represented the lower limit of detection. By using serum dilutions of 1 : 20, the system could therefore be used to reliably semiquantify IgG1 and IgG4 concentrations over a range that was 5× higher or 20× lower than those normally found in serum. The imaging system also allowed detection of bands that appeared poorly resolved to the naked eye.

With the image acquisition of the chemiluminescent signals, longer exposure times were found to increase the sensitivity but also the likelihood of image saturation. To overcome this problem, short exposure times were used but repeatedly. This increased the sensitivity while keeping background noise to a minimum. The optimal exposure settings were 32 × 1 min exposures for IgG1 blots and 32 × 20 s exposures for IgG4 blots.

The coefficients of variation for repeated measurement of bands over the whole molecular weight range was typically around 10%, with a maximum of 15% and a

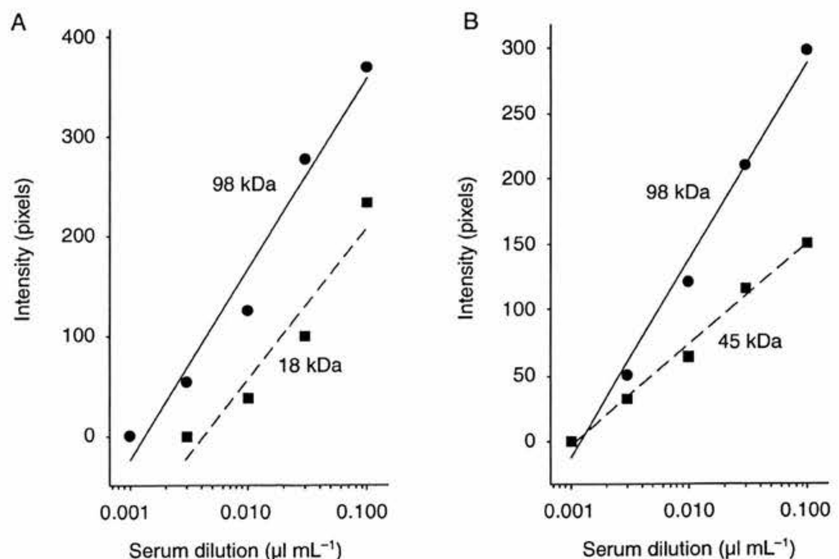

Figure 1. Effect of serum dilution on band intensity. A reference serum sample from a healthy dog was used and the intensity of each band was measured using image analysis software. (A) Standard curves generated for IgG1 against two commonly detected proteins with molecular weights of 98 and 18 kDa. (B) Standard curves generated for IgG4 against two commonly detected proteins with molecular weights of 98 and 45 kDa. Linearity was obtained with serum dilutions ranging between 1 : 10 and 1 : 1000.

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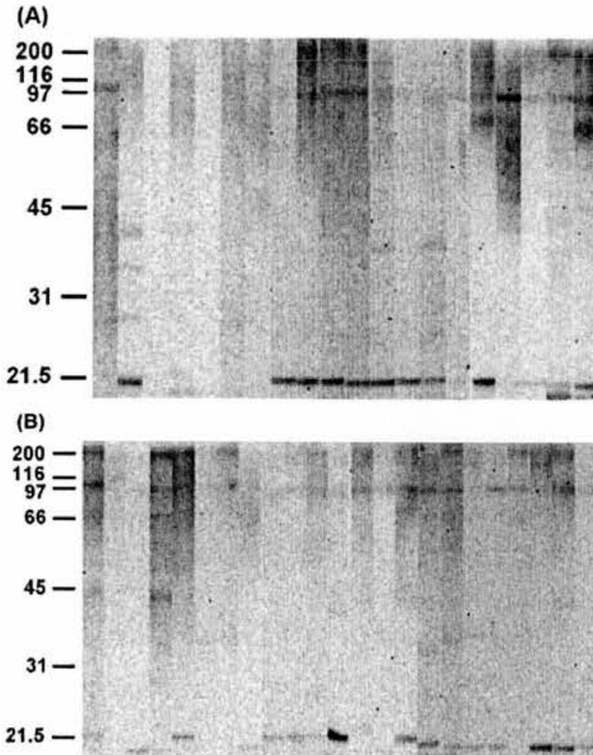

Figure 2. Binding of canine IgG1 to separated proteins of *D. farinae* in 20 healthy dogs (A) and 23 atopic dogs (B). Each strip represents one dog. The most common bands observed in both groups had molecular weights of approximately 18 and 98 kDa. A band at approximately 180 kDa was more prevalent in the atopic group.

minimum of 3%. These were considered satisfactory for semiquantitative analysis.

Dermatophagoides farinae-specific IgG1 and IgG4 subclass responses in atopic and healthy dogs

The IgG1 chemiluminescent blots of all dog sera from the two groups are shown in Fig. 2. In both healthy and atopic dogs, two main bands were visible with molecular weights of approximately 18 and 98 kDa, but some less well resolved higher molecular weight proteins were also seen. The intensity and molecular weight data from these blots, derived from the image analysis software, is shown in Fig. 3. The intensity of each band represents the magnitude of the IgG1 response to that particular protein. The most commonly recognized allergen was the 98 kDa protein, followed by the low molecular weight 18 kDa antigen. In addition, a protein with an approximate molecular weight of 180 kDa was also seen in both groups, but was significantly more prevalent in the atopic group ($P = 0.005$). The total number of bands recognized per dog did not differ significantly between the two groups ($P = 0.99$) and varied between one and eight in the healthy group and two and six in the atopic group, with median band numbers of 3.5 and 3, respectively. The sum of all the band intensities in each group (i.e. the total IgG1 response to all *D. farinae* allergens) was 85 616 in the healthy group and 65 094 in the atopic group, but this difference was not significant ($P = 0.6879$). There were no significant differences between the groups in band intensities for the 18, 98 and 180 kDa proteins.

The IgG4 chemiluminescent blots of all dog sera from the two groups are shown in Fig. 4, with the image analysis data graphed in Fig. 5. A larger number of bands were seen compared to IgG1, with proteins ranging in molecular weights from 18 to 180 kDa. The number of bands recognized did not differ between the two groups ($P = 0.5975$) and varied between one and nine in the healthy group and one to 11 in the atopic group, with median band numbers of six and four, respectively. The sum of all the band intensities in each group (i.e. the total IgG4 response to all *D. farinae* allergens) was 106 700 in the healthy group and 87 813 in the atopic group ($P = 0.3123$). There were no significant differences in band intensity for individual proteins between the two groups.

Discussion

The semiquantitative Western blot analysis system developed and validated for investigation of *D. farinae*-specific IgG subclass responses showed that the four monoclonal

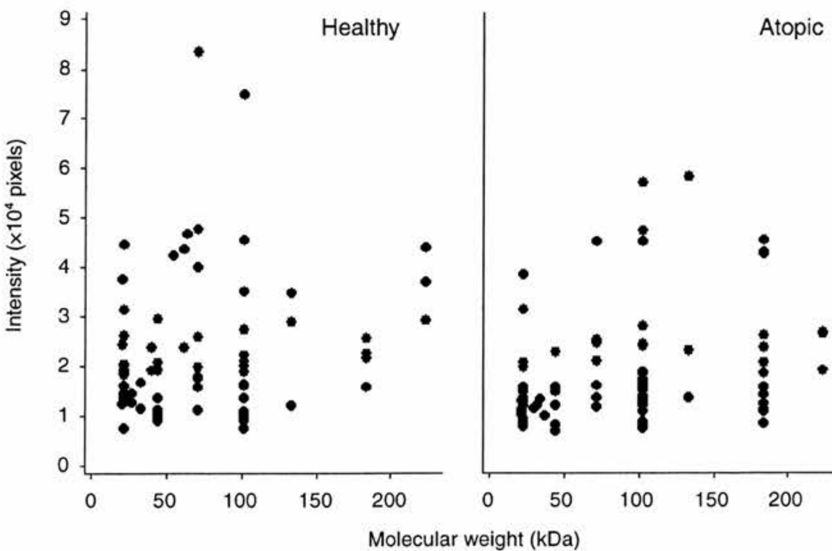

Figure 3. Plot of band molecular weight against band intensity in healthy and atopic dogs, as measured by the image analysis software. Each dot represents an IgG1 response to a *D. farinae* protein in an individual dog. The most commonly recognized proteins in both groups had molecular weights of approximately 18 and 98 kDa. A protein with a molecular weight of approximately 180 kDa was significantly more prevalent in the atopic group.

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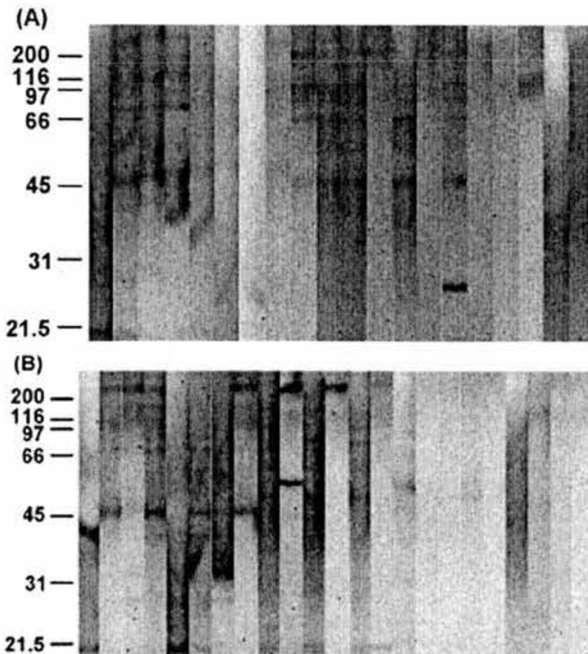

Figure 4. Binding of canine IgG4 to separated proteins of *D. farinae* in 20 healthy dogs (A) and 23 atopic dogs (B). Each strip represents one dog. Bands with molecular weights of 18, 45, 66, 98, 130 and 180 kDa were recognized; any above 200 kDa could not be evaluated because they were outwith the range of the molecular weight markers.

antibodies specific for the IgG subclasses could recognize the appropriate antibodies in immunoblots using a whole IgG preparation. The much stronger signals obtained for IgG1 and IgG4 than for IgG2 and IgG3 do not reflect the serum levels of the four subtypes as reported by Mazza,[30] where IgG1 and IgG2 were shown to have at least nine-fold higher concentrations than IgG3 and IgG4. One explanation for this potential discrepancy is that the whole IgG used in these validation experiments may have had concentrations of subclasses within it that do not mirror serum concentrations. Alternatively, the results may indicate that the monoclonal antibodies used for IgG2 and IgG3 were less able to bind to immunoblots compared to the ELISAs used by Mazza,[30] leading to lower signal strengths. This would lower the sensitivity of the immunoblotting system to detect IgG2 and IgG3 responses. Further studies using ELISAs to precisely quantify the subclass concentrations in the whole IgG preparation are required to clarify this issue.

When the monoclonal antibodies were used to detect *D. farinae*-specific subclass responses, a similar, very low sensitivity was obtained for IgG2 and IgG3. This could be due to poor binding of the reagents in this type of system as suggested above, or it could reflect the possibility that IgG2 and IgG3 responses are minimal against environmental antigens such as those from *D. farinae*, as suggested by Day.[17] Further evaluation of the reagents is required to answer this question.

The generation of linear standard curves from the IgG1 and IgG4 responses to *D. farinae* proteins allowed semi-quantitative analysis of the antibody concentrations in canine serum. It has been shown previously that this type of image analysis system can be used to measure the total IgG response to antigens in Western blots,[32] but this is the first report of such a system being used to measure IgG subclass responses.

The IgG1 and IgG4 subclass responses to various antigens of the house dust mite *D. farinae* were similar in healthy and affected dogs, both in terms of the number of bands recognized and of their molecular weights. The only significant difference detected between the two groups was the increased frequency of recognition of a 180 kDa protein in the atopic dogs. These results indicate that *D. farinae* produces a number of proteins that are recognized by the canine immune system, both in healthy and in atopic dogs, and are in accordance with previous findings.[12,32] However, studies by Day *et al.*[17] detected antigen-specific IgG antibodies against crude *D. farinae* extracts in only one of 11 normal dogs. The reason for this discrepancy is unknown but it could be due to greater sensitivity of the chemiluminescent method compared to ELISAs. It might also reflect technical, geographical, environmental or

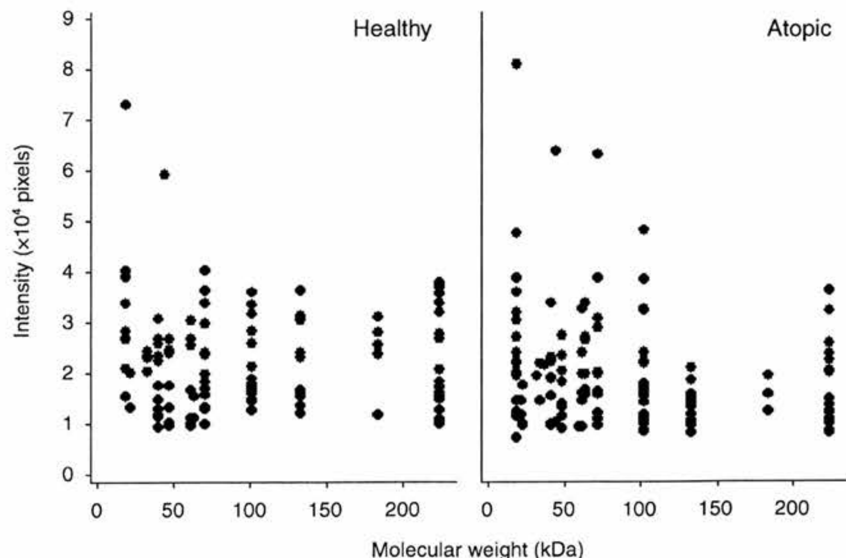

Figure 5. Plot of band molecular weight against band intensity in healthy and atopic dogs, as measured by the image analysis software. Each dot represents an IgG4 response to a *D. farinae* protein in an individual dog. The most commonly recognized proteins had molecular weights of 18, 45, 66, 98, 130 and 180 kDa. No significant differences were seen between the two groups.

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gene pool differences in the healthy dogs included in the respective studies.

In terms of the proteins recognized, the IgG1 and IgG4 response to an antigen with a molecular weight of approximately 98 kDa is likely to be the high molecular weight allergen Der f 15 that is recognized by IgE in atopic dogs.[33–35] IgG1 also recognized a low molecular weight protein of approximately 18 kDa, whereas IgG4 more commonly recognized an antigen with a molecular weight of approximately 45 kDa. A protein of the same molecular weight is also recognized by IgE in immunoblots.[35] These findings suggest that some allergens can stimulate different subclass responses in individual dogs. The significance of this finding is currently unknown but it warrants further study.

The identity of the 180 kDa protein recognized more commonly in atopic dogs is currently unknown. However, proteins of similar high molecular weight have been reported as allergens in canine atopic dermatitis.[36] The role of this protein requires further investigation and it would be premature to conclude a role of it in the pathogenesis of atopic dermatitis.

The strength of the IgG1 and IgG4 responses was generally slightly higher in the healthy controls than in the atopic dogs, both overall and for the most intense bands. Although there was no significant difference between the groups, suggesting no biological relevance, a study with a larger number of dogs in each group is required to establish that no genuine and relevant difference exists. Hill *et al.*[16] reported that total IgG concentrations were significantly higher in atopic and parasitized dogs compared to healthy controls. However, that study did not measure allergen-specific IgG, or IgG subclasses, and the antibody response could have been directed against a range of antigens, including those derived from staphylococci and *Malassezia* organisms.[37,38]

Although it is not possible to accurately compare the strength of the IgG1 response to the IgG4 response because the exact concentrations and affinities of the monoclonal antibodies were unknown, there is some evidence to suggest that the IgG4 response to *D. farinae* antigens was stronger. The IgG4 concentration in normal dog serum is nine times less than IgG1 and yet in the current experiments, the signals on the blots were much stronger (compare Figs 2 and 4), even when using shorter exposure times.

Taken together, the results of this and previous studies suggest that *D. farinae*-specific IgG and IgG subclasses do not play a major role in the pathogenesis of canine atopic dermatitis. Such responses occur in both healthy and atopic dogs to the vast majority of antigens and do not appear to be either protective or pathogenic. Some of the proteins recognized (98 kDa, 45 kDa) are regarded as major allergens in relation to IgE production and yet were found in both the healthy and the atopic groups in equal numbers and intensity. Whether the 180 kDa protein has a more specific role remains to be determined. These findings fit a hypothesis in which the production of IgG, and its subclasses, against the majority of *D. farinae* antigens is merely a result of recognition of a foreign protein presented to the immune system in relatively large quantities. They also accord with the concept of *D. farinae* antigens acting as 'danger' signals to the immune system, thus provoking an antibody response even though the molecules themselves appear to be harmless. In view of this, it seems likely that the production of IgE, and the activation of allergen-specific T lymphocytes, are the most important immunological events in canine atopic dermatitis.

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Résumé Un système semi-quantitatif de western blot a été développé et validé pour évaluer les sous-classes d'IgG spécifiques observées au niveau de protéines de *Dermatophagoides farinae* séparées par électrophorèse chez des chiens sains et des chiens atopiques.

Les deux groupes ont montré des réponses IgG1 et IgG4 similaires mais il a été difficile de détecter des réponses IgG2 et IgG3. Les bandes les plus facilement reconnues dans les deux groupes étaient des antigènes de 18 et 98 kDa pour les IgG1 et de 18, 45, 66, 98, 130 et 180 kDa pour les IgG4. Le nombre de bandes reconnues par chien n'était pas statistiquement significatif, mais plus de chiens atopiques présentaient une réponse IgG1 à la protéine de 180 kDa protein. Les réponses IgG1 et IgG4 étaient un peu plus élevées dans le groupe sain, mais sans différence significative. Ces résultats suggèrent que certains antigènes produits par *Dermatophagoides farinae* peuvent provoquer la synthèse de différentes sous-classes d'anticorps. Cependant, comme la plupart de ces anticorps sont retrouvés chez les chiens sains et les chiens atopiques, il s'agit probablement une reconnaissance de protéines étrangères par le système immunitaire plutôt qu'ils jouent un rôle dans la pathogénie de la dermatite atopique. Le rôle joué par l'antigène de 180 kDa nécessite des études supplémentaires.

Resumen Se desarrolló un análisis semi-cuantitativo de western blot utilizando quemoluminiscencia y se demostró su eficacia para evaluar la respuesta en subclases de inmunoglobulinas específicas de antígeno en perros sanos y atópicos, frente a proteínas de *Dermatophagoides farinae* separadas mediante electroforesis.

Ambos grupos respondieron de forma similar con IgG1 e IgG4 específicas frente a *D. farinae* para múltiples antígenos, pero IgG2 e IgG3 fueron difíciles de detectar. Las bandas mas comúnmente reconocidas en ambos grupos fueron antígenos de 18 y 98 kDa para la IgG1 y de 18, 45, 66, 98, 130 y 180 kDa para la IgG4. El número de bandas reconocido no varió de forma significativa entre los perros, pero significativamente un mayor número de perros atópicos desarrolló una respuesta de IgG1 frente a la proteína de 180 kDa. En conjunto, las respuestas de IgG1 e IgG4 específicas frente a *D. farinae* fueron ligeramente mas pronunciadas, pero no significativamente diferentes, en el grupo sano. Los resultados sugieren que algunos antígenos producidos en *D. farinae* pueden inducir respuestas con diferentes subclases de inmunoglobulinas. Sin embargo, como la mayoría de las respuestas se observaron tanto en perros sanos como atópicos, es presumible que representen simplemente el reconocimiento de proteínas extrañas presentadas al sistema inmune, más que estar implicadas en la patogenia de la dermatitis atópica. El papel de la proteína de 180 kDa merecería un estudio más detallado.

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Zusammenfassung Ein semi-quantitatives Chemilumineszenz Western-Blot Analyse System wurde entwickelt und validiert, um die Antwort von antigen-spezifischen IgG Unterklassen auf elektrophoretisch separierte Proteine von *Dermatophagoides farinae* bei gesunden und atopischen Hunden zu evaluieren. Beide Gruppen entwickelten ähnliche *D. farinae*-spezifische IgG1 und IgG4 Antworten auf multiple Antigene, während IgG2 und IgG3 Antworten schwierig festzustellen waren. Die am häufigsten erkannten Banden entsprachen in beiden Gruppen 18 und 98 kDa Antigenen für IgG1 und 18, 45, 66, 98, 130 und 180 kDa für IgG4. Die Anzahl der Banden, die von jedem Hund erkannt wurden, war nicht signifikant unterschiedlich; allerdings zeigten signifikant mehr atopische Hunde eine IgG1 Antwort auf ein 180 kDa Protein. Die gesamten *D. farinae*-spezifischen IgG1 und IgG4 Antworten waren in der gesunden Gruppe etwas höher, aber nicht signifikant unterschiedlich. Die Ergebnisse weisen darauf hin, dass einige Antigene, die von *Dermatophagoides farinae* produziert werden, Antworten verschiedener Unterklassen induzieren können. Da jedoch die Mehrheit dieser Immunantworten bei gesunden wie auch bei atopischen Hunden gesehen werden, ist es wahrscheinlicher, dass diese nur die Erkennung von Fremdproteinen durch das Immunsystem repräsentieren anstatt einen Einfluss der Pathogenese der atopischen Dermatitis. Die Rolle des 180 kDa Antigens sollte weiter untersucht werden.

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1.9

Dermatophagoides farinae-specific IgG subclass responses in atopic dogs undergoing allergen-specific immunotherapy

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Summary

The molecular and immunologic mechanisms involved in successful allergen-specific immunotherapy (ASIT) have not been completely elucidated. The aim of this study was to characterise the changes in *Dermatophagoides farinae*-specific total IgG and IgG subclasses during ASIT of dogs with atopic dermatitis.

Twenty-one dogs with *D. farinae* hypersensitivity were treated with alum-precipitated vaccines for 9 months. Serum samples were collected before and after 3, 6 and 9 months of therapy and used to probe Western blots containing separated proteins of *D. farinae*. IgG responses were detected using a polyclonal antibody and a colorimetric substrate, whereas IgG subclasses were detected using a panel of monoclonal anti-IgG antibodies and chemiluminescence. The blots were analysed using a semi-quantitative digital image analysis system that provided the number and molecular weight of bands, as well as their intensity, which was related to IgG concentration. Prior to ASIT, all dogs showed an IgG, IgG1 and IgG4 response to multiple proteins of different molecular weights, the most common being 98 kDa and 44 kDa. There was virtually no detectable IgG2 or IgG3 response. During ASIT, the total IgG, IgG1 and IgG4 response to *D. farinae* antigens varied widely between dogs and could increase, decrease, fluctuate or remain the same, but there was no induction of IgG2 or IgG3 antibodies. There were no significant increases in total IgG or IgG subclass responses in dogs showing a complete or partial response to ASIT. However, dogs showing no response to ASIT had significantly higher *D. farinae*-specific total IgG levels prior to the start of therapy, compared to dogs that responded to ASIT.

Introduction

Allergen-specific immunotherapy (ASIT) is the practice of administering gradually increasing quantities of an allergen extract to an allergic subject to ameliorate the symptoms associated with subsequent exposure to the causative allergen.[1,2] Since the first reports of ASIT in humans[3,4] and in the dog,[5] many studies, and a large body of clinical observations by veterinary dermatologists, have suggested that ASIT can be effective in controlling the signs of atopic dermatitis (AD) in dogs.[2,6,7] Most of these data are either anecdotal or originate from questionnaire-based studies and, to date, there has been only one placebo-controlled study that has investigated this form of treatment in dogs.[8] Collectively, this body of information and literature has resulted in efficacy claims ranging from 50% to 100%, with a response usually being defined as an improvement in clinical signs of at least 50%.[2]

Two main forms of ASIT vaccines are currently used in dogs: aqueous vaccines are favoured in North America whereas aluminium-adsorbed vaccines are commonly used in Europe. A comparison between these vaccine types of their true efficacy is not possible because different studies quote differences in allergen extracts, the number of allergens used, the concentration of allergens, the frequency of administration, as well as symptom scores which are easily influenced by other factors such as infections. However, one potential advantage of aluminium-adjuvanted vaccines is the more rapid development of

high titre, and long-lasting, antibody responses after primary immunisation.[9] The mechanism of action of aluminium adjuvants likely involves the formation of a depot, increasing targeting of antigens to antigen-presenting cells, and non-specific activation of the immune response.[10]

Although the clinical efficacy of ASIT is well documented in humans and dogs, the molecular and immunological mechanisms involved are incompletely understood. Current evidence from rodent and human studies suggests that ASIT exerts effects on several aspects of the immune system, including modulation of allergen-specific B cells as well as T-cell responses. Studies on the effect of ASIT have demonstrated reduced basophil reactivity to allergens,[11] deviation of Th2-cytokine responses to allergens in favour of a Th1 response,[12–15] and the induction of IL-10-producing regulatory T cells.[16–18] In addition, changes in serum antibody titres in response to ASIT have been described, mostly as increases in allergen-specific IgG antibodies, particularly of the IgG1 and IgG4 isotypes.[19,20] Allergen-specific IgG produced in response to ASIT has been termed 'blocking antibody' because it has been proposed that competition with IgE may prevent successful activation of mast cells.

To the authors' knowledge, only two studies investigating changes in IgG concentrations have been reported in dogs.[21,22] In the first of these reports, increases in allergen-specific IgG antibodies to various pollens were reported during ASIT with aqueous allergens.[21] The second report showed that concentrations of total IgG1 (i.e. not allergen-specific) could increase following ASIT using adjuvanted vaccines.[22] However, no studies have reported changes in IgG antibodies specific for antigens derived from the house dust mite *Dermatophagoides farinae*, a major cause of AD in dogs.[23–26]

The aim of this study was to use a semi-quantitative blot analysis system[27] (C. Hou, M.J. Day, T.J. Nuttall, P.B. Hill, unpublished data) to investigate the changes in total IgG, and the subclasses IgG1, IgG2, IgG3 and IgG4, to separated antigens from *D. farinae*, during ASIT with aluminium-adsorbed vaccines. Our hypothesis was that allergen-specific IgG antibodies would increase after the administration of allergen-specific immunotherapy in dogs with AD.

Materials and methods

Study population

Serum samples used in this study were obtained from 21 dogs with AD taking part in another study to evaluate the efficacy of two different allergen-specific immunotherapy (ASIT) protocols. Details of the inclusion and exclusion criteria, study design, clinical assessment and scoring, use of concurrent medications and analysis of treatment efficacy, will be reported elsewhere.[28] Briefly, the diagnosis of AD was based on a combination of a compatible history and clinical signs,[29] exclusion of other pruritic skin diseases (e.g. ectoparasite infestation and adverse food reaction), and the presence of at least one positive reaction either in an intradermal test or a commercially available allergen-specific IgE assay (Allercept™, Heska Corporation, Fort Collins, CO, USA). All dogs included in the present study had a positive reaction to *D. farinae* at a dilution of 1/1000 w/v on an intradermal test; test sites were scored from 0 to 4 compared to the positive (1/100 000 w/v histamine) control and the negative (diluent) control, with reactions ≥ 2 being considered positive. Multiple coat brushings and skin scrapings were used to eliminate the possibility of an ectoparasite infestation. All dogs were also placed on a rigorous flea and sarcoptic mange control programme using selamectin (Stronghold® spot-on, Pfizer Animal Health, Sandwich, UK) for 6 weeks before the start of the study and at monthly intervals throughout the study. Dogs that experienced complete resolution of clinical signs after treatment were excluded. A 6-week novel ingredient home-cooked diet trial was conducted to eliminate the possibility of adverse food reactions. Concurrent staphylococcal pyoderma was managed with oral cephalexin and topical treatment with either benzoyl peroxide (Paxcutol®, Virbac, UK) or 10% ethyl lactate (Etiderm®, Virbac). Concurrent *Malassezia* infection was treated with 2% miconazole/2% chlorhexidine shampoo (Malaseb®, Leo Animal Health, UK). No glucocorticoids were given for 3 months prior to the initiation of ASIT.

Ten dogs received a standard immunotherapy protocol (Artuvetrin® Therapy, ARTU, The Netherlands), as used in the dermatology clinic at the Royal (Dick) School of Veterinary Studies in Edinburgh (Table 1.9.1). This involved administration of an allergen mixture comprising up to eight allergens, at least one of which was *D. farinae* which accounted for 2.5–10%

Table 1.9.1 Standard-dose and low-dose protocols for allergen-specific immunotherapy using aluminium-adsorbed allergens administered by subcutaneous injection

	Dose of allergen extract (ml)	
Week	Standard-dose ASIT	Low-dose ASIT
0	0.2	0.1
2	0.4	0.1
4	0.6	0.1
6	0.8	0.1
9	1.0	0.1
12	1.0	0.1
16	1.0	0.1
20	1.0	0.1
Every 4 weeks	1.0	0.1

v/v of the allergen mixture. Eleven dogs received an alternative protocol in which the frequency of injections was identical but the maintenance dose of allergen was only 1/10 that of the standard protocol (Table 1.9.1). The precise concentration of allergens is not provided by the manufacturer.

Dogs were assessed for pruritus by the owners using a 0–5 point behaviour-based scale and for skin lesions by a single investigator using a modified canine atopic dermatitis extent and severity index (CADESI).[28] Assessments took place on the day the ASIT was started and after 3, 6 and 9 months (90, 180 and 270 days) of ASIT. A complete response to ASIT was defined as a dog whose pruritus score had fallen to zero by the end of the study without the need for additional anti-pruritic medication. A partial response to ASIT was defined as a dog whose pruritus score was lower at the end of the 9-month treatment period compared to the beginning. No response to ASIT was defined as a dog whose pruritus score was the same or higher after 9 months of treatment compared to the beginning. Glucocorticoid therapy was permitted for the first 6 months of the study, but not during the final 3 months of ASIT.

Serum samples

Serum samples were to be collected from each dog before and after 3, 6 and 9 months of ASIT. However, due to unavoidable logistical and technical circumstances, serum samples could not be collected at every time point in every dog. All dogs were sampled prior to the start of ASIT but some only had serum samples taken at one or two of the remaining three time points. There were 4/24 missing data points in the complete response group, 5/20 in the partial response group and 5/40 in the no response group.

All samples were obtained with the owner's consent. As the samples were to be used for measurement of allergen-specific antibody responses, which represented diagnostically and therapeutically useful information; UK Home Office approval was not required. After collection, serum was separated by centrifugation and stored in aliquots at –20°C until used.

D. farinae *extract*

Lyophilised whole-body *D. farinae* crude extract (Greer Laboratories, Lenoir, NC, USA) was reconstituted to 1 mg/ml in sterile phosphate-buffered saline (PBS, pH 7.5) and 200-μl aliquots were stored at –20°C until used.

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

SDS-PAGE was performed according to the method of Laemmli[30] using a 10% Tris-glycine polyacrylamide (ProtoGel®, National Diagnostics, UK) separating gel and 4% stacking gel in a discontinuous buffer system containing 25 mM Tris-HCl (Trizma® hydrochloride; Sigma-Aldrich, Dorset, UK), 0.2 M glycine (Fisher Scientific, Loughborough, UK) and 0.1% SDS (Fisher Scientific), pH 8.3. A total of 50 μl (for total IgG colorimetric blots) or 100 μl (for IgG subclass chemiluminescent blots) of *D. farinae* extract, and 0.5 μl of the molecular weight markers, were diluted 1:1 and 1:20, respectively, with reducing sample buffer containing 125 mM Tris-HCl, 4% SDS, 20% glycerol (BDH Chemicals, Poole, UK), 0.005% bromophenol blue (BDH) and 10% 2-mercaptoethanol (Sigma-Aldrich) and heated at 95°C for 5 minutes. The extract was then loaded into one broad well across the top of the gel alongside a lane containing the molecular weight markers and the electrophoresis was run at 200 V for 40 minutes according to the manufacturer's recommendations (Mini-Protean II®; Biorad, Hercules, USA).

Western blots

Separated *D. farinae* proteins were transferred to polyvinylidene fluoride (PVDF) microporous membranes (Millipore Immobilon™-P Transfer Membrane, Mil-

lipore Corporation, Bedford, MA, USA) in a semi-dry electrophoretic transfer cell (Trans-Blot® SD, Biorad) using 0.3 M Tris-HCl/10% methanol (pH 10.4) on the lower anode, 25 mM Tris-HCl/10% methanol (pH 10.4) on the upper anode and 25 mM Tris-HCl/40 mM 6-amino-n-hexonic acid/10% methanol (pH 9.4) on the cathode according to the manufacturer's instructions. The transfer was run at 80 mA per minigel for 1 hour, after which the membranes were dried and stored at 4°C. The quality of transfer was checked by staining gels and molecular weight standards blotted onto the membrane with Coomassie brilliant blue R-250 (BDH).

Antibodies

To assess total IgG responses to separated antigens from *D. farinae*, a polyclonal horseradish peroxidase-conjugated goat anti-dog IgG (Bethyl Laboratories Inc., Montgomery, TX, USA) was used. The specificity of this reagent for IgG is verified by the manufacturer and has been documented in previous studies.[27,31] Potential cross-reactivity of the reagent with canine IgE is not relevant in colorimetric immunoblots because the method is not sensitive enough to detect the low concentrations of IgE present, even if specific anti-IgE reagents are used.[31]

To assess IgG subclass responses to separated antigens from *D. farinae*, a panel of four mouse monoclonal antibodies (mAbs) specific for the subclasses of canine IgG were used. The preparation and characterisation of these mAbs has been described.[32–35] mAb B6 has restricted specificity for canine IgG1 and IgG3, but reacts specifically with IgG1 in serological and immunohistochemical assays. mAb E5 is specific for canine IgG2 and antibody A3G4 is specific for canine IgG3. mAb A5 has restricted specificity for canine IgG2 and IgG4 but reacts specifically with IgG4 in serological and immunohistochemical assays. The specific activity of the subclass-restricted mAbs B6 and A5 is probably due to conformational differences between the target IgG subclasses in solid-phase assays versus other serological and immunohistochemical techniques.[33,35] mAbs A5, E5 and B6 were used as 45% ammonium sulphate precipitates of tissue culture supernatants, and A3G4 was used as a tissue culture supernatant. The applicability of these mAbs in Western blotting was confirmed by probing a commercial preparation of canine IgG purified by ion-exchange chromatography (Sigma-Aldrich).

Immunoblotting

Blotting membranes with transferred separated *D. farinae* proteins were cut longitudinally into 4-mm wide strips to allow probing by individual dog sera. Strips were placed in individual lanes of an eight-channel incubation tray (Biorad), blocked with 5% skimmed milk in Tris-buffered saline (TBS, pH 7.5) containing 20 mM Tris-HCl and 0.5 M NaCl (Sigma-Aldrich) for 1 hour at room temperature, and washed with 0.1% Tween 20 (Fisher Scientific)/TBS (TTBS) three times for 5 minutes. All sera and primary and secondary reagents were diluted with dilution buffer (1% skimmed milk/TTBS) to a final volume of 1 ml and to concentrations that had been determined in previous studies to yield strong bands against a clear background[27] (C. Hou, M.J. Day, T.J. Nuttall, P.B. Hill, unpublished data).

For total IgG immunoblots, duplicate strips were probed with individual dog sera at 1/100 for 1 hour, followed by a 1-hour incubation with the horseradish peroxidase-conjugated goat anti-dog IgG at 1/1000. After further washing with washing buffer, the strips were developed for 1 minute with 3,3'-diaminobenzidine (DAB) peroxidase substrate (Vector Laboratories Inc., Burlingame, CA, USA), prepared according to the manufacturer's recommendations. The strips were air-dried overnight prior to analysis.

For detection of IgG subclass responses, duplicate strips were probed for 1 hour with dog sera diluted to 1/20, followed by a 1-hour incubation with one of the four IgG subclass-specific mAbs diluted to 1/500. After washing, a horseradish peroxidase-conjugated bovine anti-mouse IgG (Bethyl Laboratories) diluted to 1/1000 was added for another hour followed by thorough washing. The surface of each strip containing the detected proteins was then covered for 1 minute with a chemiluminescent luminal solution (ECL, Amersham-Pharmacia Biotech, Little Chalfont, UK), used for its higher sensitivity. The excess liquid was then drained off prior to immediate image acquisition as described below.

Digital image acquisition and semi-quantitative analysis

Strips probed with the polyclonal anti-canine IgG and developed with the colorimetric substrate DAB, along with the molecular weight standards, were aligned and

digitally scanned using a flatbed scanner (Epson Perfection 1650, Hemel Hempstead, UK) set at 16-bit grey scale. For IgG subclass-specific blots, developed with the chemiluminescent substrate ECL, the strips were wrapped in cling film and placed protein side down on a Kodak Digital Science™ Image Station 440CF (Kodak, Rochester, NY, USA) along with the Coomassie blue-stained molecular weight standards. In this system, the images are acquired with a charge coupled device (CCD) camera. Three exposures, each lasting 5 minutes, were used to eliminate the possibility of image saturation.

The scanned and acquired images were then imported into image analysis software (Kodak Digital Science™ 1D Image Analysis Software, Kodak, USA) to detect the bands on the blots. This software can determine, without operator bias, the molecular weight of bands by analysing their positions relative to the molecular weight standards as well as the signal magnitude of individual bands. We have shown in previous studies that this system can be used for semi-quantitative analysis of antibody concentrations because a linear standard curve is obtained when band intensity is plotted against log serum dilutions[27] (C. Hou, M.J. Day, T.J. Nuttall, P.B. Hill, unpublished data). Furthermore, the coefficients of variation obtained from repeated assay of samples are approximately 10–20%, and the conditions used do not lead to saturation effects[27] (C. Hou, M.J. Day, T.J. Nuttall, P.B. Hill, unpublished data). The sum of the band intensities on each strip therefore provides an indication of the antibody response to *D. farinae* as a whole, whereas the intensity of each band provides an indication of the antibody response to individual antigens. For each dog, the final band intensity was derived from an average of each set of duplicate strips.

Statistical analysis

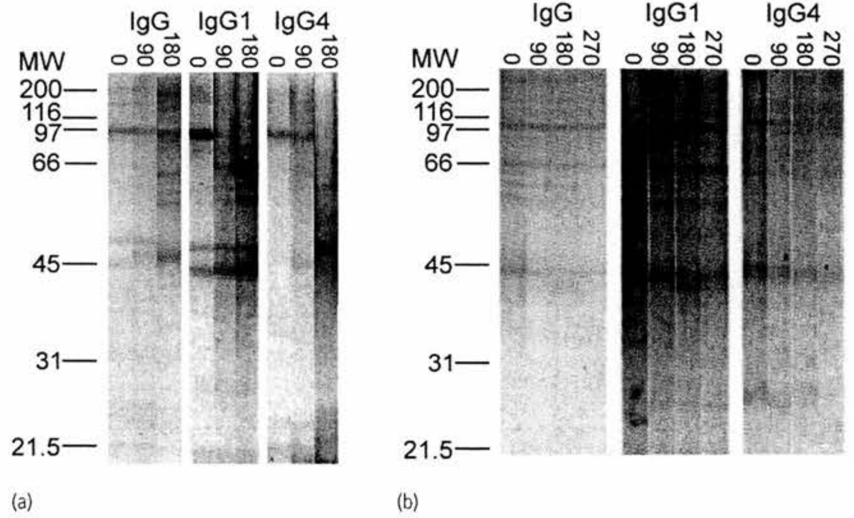
Numerical data derived from the image analysis software were exported to Graphpad Prism, Version 3.0 for Windows (Graphpad software, San Diego, USA). As antibody concentrations in dogs are not normally distributed,[36] the Kruskal–Wallis non-parametric ANOVA was used to compare antibody responses at multiple time points. If significant differences were detected by Kruskal–Wallis, comparisons between two individual time points were made by the Mann–Whitney test. The following comparisons were made: data from four time points (days 0, 90, 180 and 270) when all the dogs were combined as a single group; data from corresponding time points when dogs were divided into two groups based on those that showed no response to ASIT versus dogs that showed some response (complete or partial); data from corresponding time points when dogs were divided into three groups comprising those that showed a complete, partial or no response to ASIT; data from the four time points within each response group; and data from eight time points when dogs were divided into two groups based on those that received the standard ASIT protocol versus those that received the low-dose ASIT protocol. A p value of < 0.05 was considered to be significant.

Results

All the dogs had detectable levels of *D. farinae*-specific total IgG, IgG1 and IgG4 prior to the start of, and during, ASIT (Figure 1.9.1). There was no detectable response by IgG2 and IgG3 antibodies, despite attempts to increase the sensitivity of the assay by increasing reagent concentrations and incubation times (data not shown). Both total IgG, and the subclasses IgG1 and IgG4, recognised multiple proteins from *D. farinae* with the most visually obvious bands having molecular weights of approximately 98 kDa and 44 kDa, both of which have been identified as major allergens[26] (Fig. 1.9.1). The 66-kDa protein was often detected in samples and it has also been classified as a minor allergen for dogs.[26]

Visual analysis of the strips from individual dogs showed that the strength of the bands varied during ASIT. However, there was no clearly obvious trend for the band intensity to increase or decrease with time. Over the course of 9 months of ASIT, individual dogs could show an obvious increase in band strength (Fig. 1.9.1a), a decrease (Fig. 1.9.1b), remain relatively static (Fig. 1.9.1c), or fluctuate. For each dog, the changes seen with total *D. farinae*-specific IgG were approximately mirrored by similar changes in IgG1 and IgG4. No consistent induction or disappearance of specific bands at any molecular weight was observed.

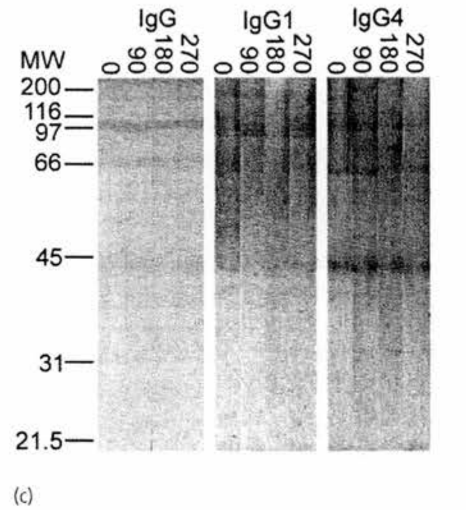
This visual impression was confirmed by the numerical data from the image analysis software which showed that, during ASIT, there was no consistent increase in the total antibody response (the sum of all the band intensities on each strip) for IgG, IgG1 or IgG4 (Fig. 1.9.2a). There was also no con-



(a)

(b)

Fig. 1.9.1 Examples of immunoblots showing total IgG-, IgG1- and IgG4-binding profiles to *Dermatophagoides farinae* extract in dogs undergoing allergen-specific immunotherapy. Serum samples were collected before and after immunotherapy for 90, 180 and 270 days. The IgG responses to *D. farinae* allergens varied widely between dogs and could increase (a), decrease (b), remain the same (c) or fluctuate. Changes in total *D. farinae*-specific IgG were usually paralleled by equivalent changes in IgG1 and IgG4. MW, molecular weight in kilodaltons (kDa).



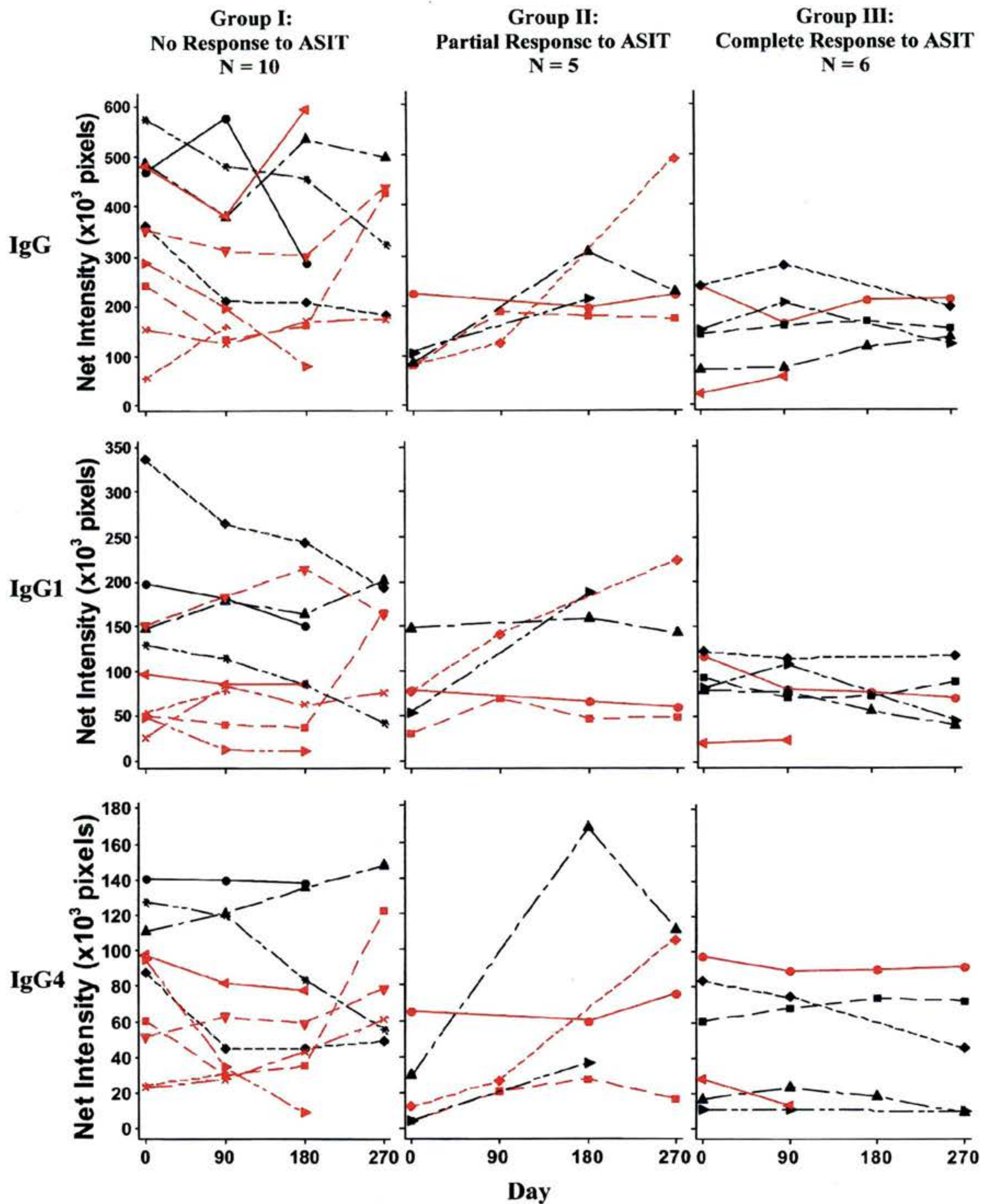
(c)

sistent change in the response to the 98-kDa band (Fig.1.9.2b) or the 44-kDa band (Fig. 1.9.2c) for any of the antibodies.

When all the dogs were considered as a single group, there was no significant difference in the total antibody response to *D. farinae* (sum of the band intensities on each strip) or the response to the 98-kDa and 44-kDa proteins at any of the four time points during ASIT for IgG, IgG1 or IgG4 ($p > 0.05$ for all nine analyses). However, when dogs that showed no response to ASIT were compared to dogs that showed a response (partial and complete response groups combined), there was a significant difference among the time points for total IgG. This difference was attributable to a significantly greater total IgG response to *D. farinae*, and the 98-kDa and 44-kDa proteins, in the group that was non-responsive to ASIT at day 0, compared to the response group at day 0. In the case of the 44-kDa band, there was also a significantly greater total IgG response in the non-responsive group at day 90 compared to the response group at day 90.

When the dogs were divided into three response groups and compared separately, there was a significant difference among time points for total IgG responses to *D. farinae*. This was due to a significantly higher IgG response in the non-responsive group at day 0, compared to the partial ($p = 0.019$) and the complete ($p = 0.011$) response groups. The total IgG responses to the 98-kDa and 44-kDa proteins were also significantly higher at day 0 in the non-responsive group compared with the complete response groups ($p = 0.023$ and $p = 0.042$, respectively).

In addition, there was also significant variation among the time points in the standard- and low-dose ASIT protocols for IgG1 against total *D. farinae* antigens. This was attributable to significantly lower IgG1



(a)

Fig. 1.9.2 *Dermatophagoides farinae*-specific total IgG, IgG1 and IgG4 antibody responses during 9 months of allergen-specific immunotherapy. (a) The total antibody response to *D. farinae* antigens (calculated as the sum of all band intensities on each immunoblot strip). The dogs are divided into three groups categorized by clinical outcome. Dogs that received the standard-dose immunotherapy protocol are plotted in black. Dogs that received the low-dose immunotherapy protocol are plotted in red.

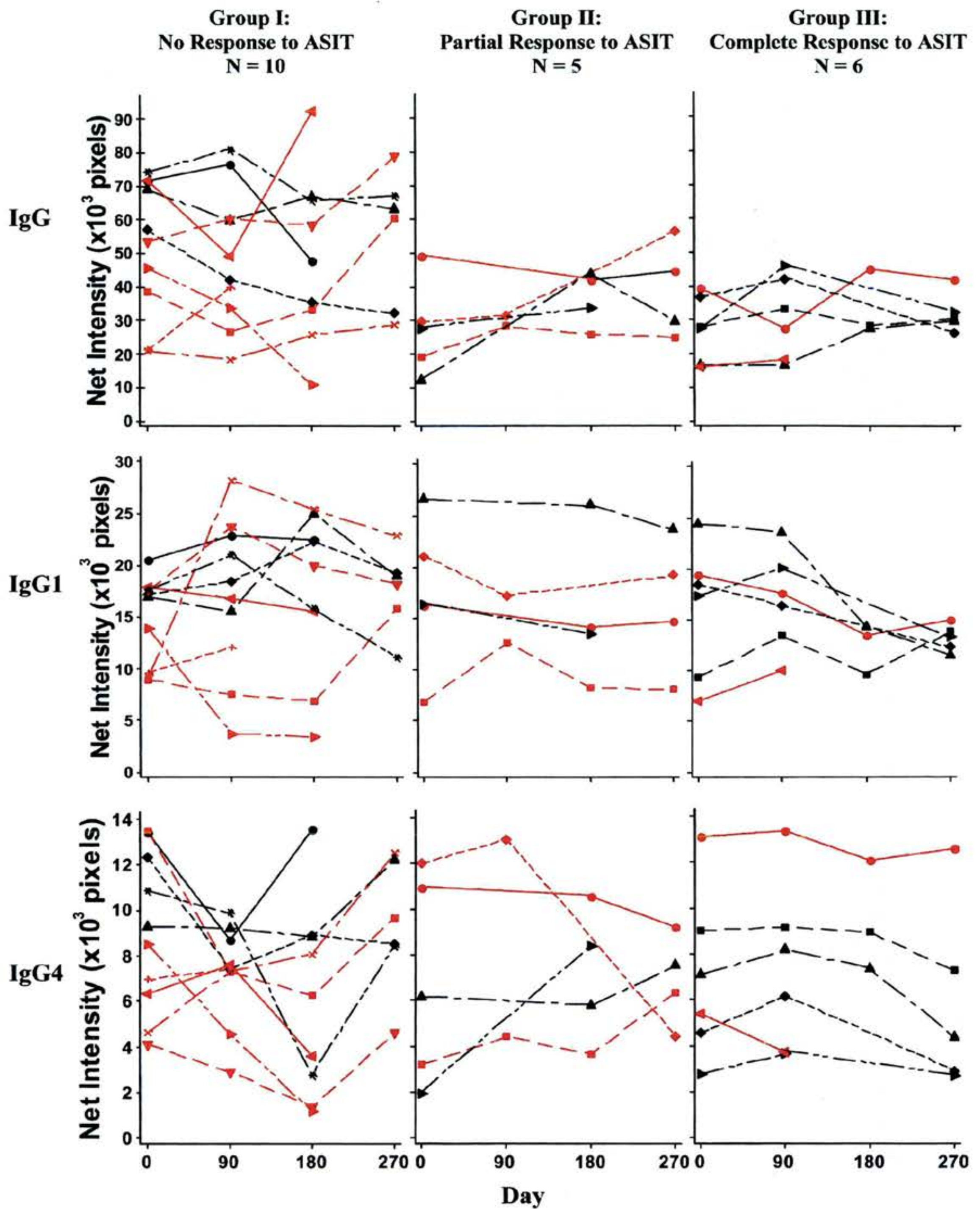
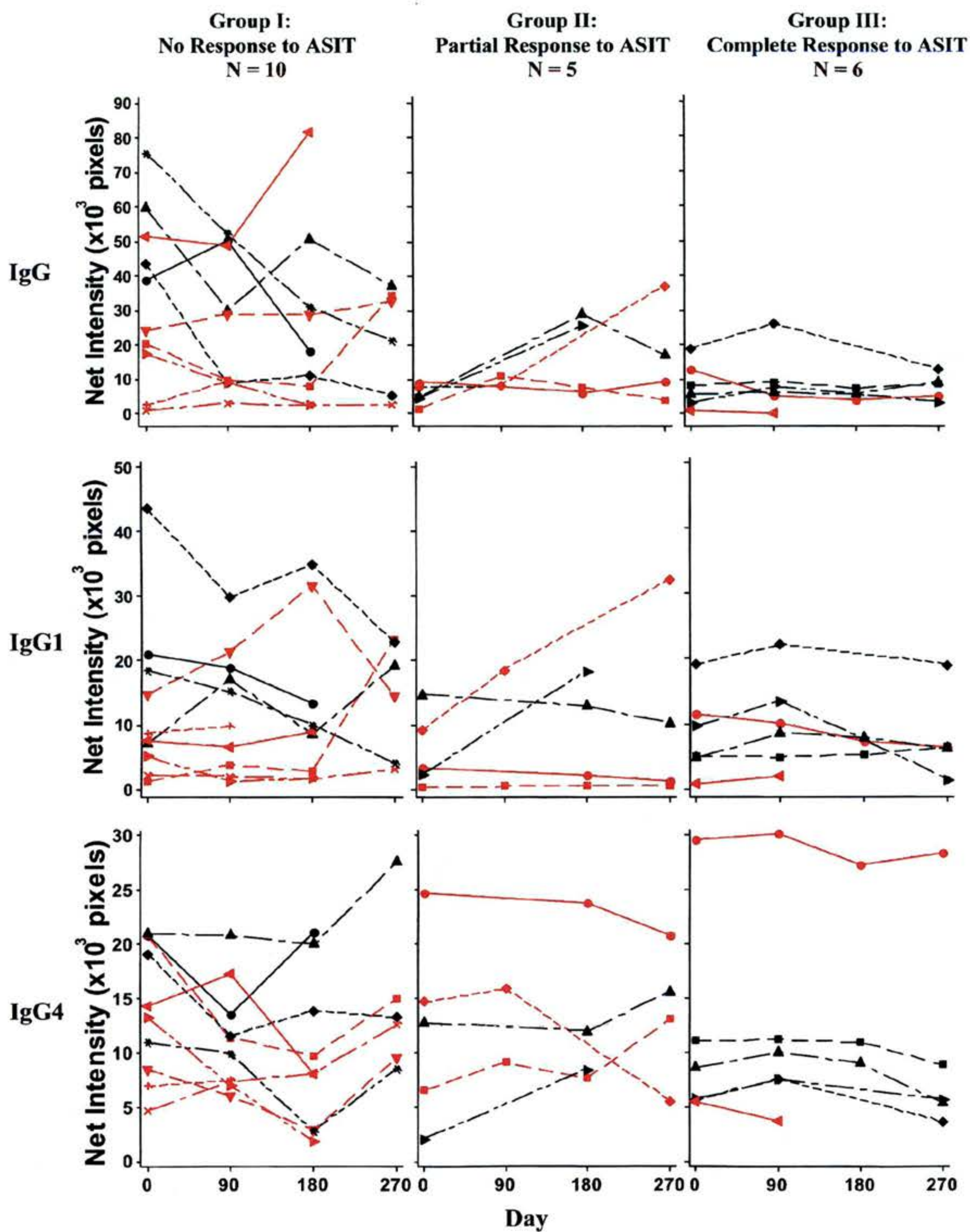


Fig. 1.9.2 *Dermatophagoides farinae*-specific total IgG, IgG1 and IgG4 antibody responses during 9 months of allergen-specific immunotherapy. (b) Antibody responses to the 98-kDa band. Dogs that received the standard-dose immunotherapy protocol are plotted in black. Dogs that received the low-dose immunotherapy protocol are plotted in red.



(c)

Fig. 1.9.2 *Dermatophagoides farinae*-specific total IgG, IgG1 and IgG4 antibody responses during 9 months of allergen-specific immunotherapy. (c) Antibody responses to the 44-kDa band. The dogs are divided into three groups categorized by clinical outcome. Dogs that received the standard-dose immunotherapy protocol are plotted in black. Dogs that received the low-dose immunotherapy protocol are plotted in red.

levels to *D. farinae* at day 0 in the dogs receiving the low-dose protocol compared with those receiving the standard dose ($p = 0.01$). There were no other significant differences for *D. farinae* or the 98-kDa or 44-kDa proteins at any time point for any other antibody between the standard-dose and low-dose protocol groups.

Discussion

In this study, we documented allergen-specific total IgG, IgG1 and IgG4 responses to various antigens of the house dust mite *D. farinae* in dogs with AD. No IgG2 or IgG3 antibodies were detected with specificity for *D. farinae*. These findings were in accordance with previous studies recently conducted in our laboratory[27] (C. Hou, M.J. Day, T.J. Nuttall, P.B. Hill, unpublished data). During ASIT, there was no consistent augmentation of the total quantity of *D. farinae*-specific IgG or IgG subclass antibodies to antigens from the mite. Levels of the antibodies could increase or fall but there was no significant increase by day 270 compared to day 0. This was the case for the total response to all the *D. farinae* proteins (measured by summing all the band intensities on each strip) as well as the response to the 98-kDa and 44-kDa proteins. These latter proteins were chosen for specific analysis because they were the strongest and most visually obvious bands on the strips. The 98-kDa band is likely to be the high molecular weight chitinase recently designated Der f 15, a major *D. farinae* allergen for dogs with AD.[23,24,26] The identity of the 44-kDa protein is currently unknown, but it may be a fragment of a larger antigen because it only appeared on blots performed with reducing buffers and not on blots performed with non-reducing buffers in an earlier study.[26]

Although not exactly parallel, the changes in IgG1 and IgG4 tended to mirror the changes seen with total IgG. The lack of significant increases in levels of allergen-specific IgG, IgG1 and IgG4 is surprising. Previous studies have demonstrated that increases in allergen-specific IgG1 and IgG4 are seen following ASIT in man.[19,20,37] In atopic dogs, concentrations of pollen-specific IgG were shown to increase following at least 6 months of ASIT using aqueous allergens.[21] An increase in total (non-allergen-specific) IgG1 was also observed in most dogs that were given ASIT using alum-precipitated vaccines.[22] The reason for the difference in the findings between our study and previous studies is not clear, but there are a number of possible explanations.

First, although administration of adjuvanted proteins should lead to a marked IgG response, some studies in humans have shown poor clinical efficacy to alum-precipitated vaccines as well as a lack of alteration in immune responses.[38,39] It is possible that the vaccines used in this study did not contain sufficient protein to induce an allergen-specific IgG response, even though they were shown to elicit an increase in total IgG1 in most of the dogs in a prior study.[22] The allergen manufacturer used in this study does not provide the specific protein content of allergens within vaccines, so it is not possible to know the precise amount of *D. farinae* that is present. Further, the quantity of *D. farinae* in each vaccine is likely to be different depending on the number of other allergens included. It is also possible that the amount of *D. farinae* antigens may vary from batch to batch, leading to further variation in specific immune responses. Evidence for the above possibility is provided by the lack of significant differences in IgG, IgG1 and IgG4 levels in dogs receiving either the standard ASIT protocol or the low-dose protocol, which involved administering 1/10 of the recommended dose. It would have been expected that dogs receiving the higher doses would have mounted a greater antibody response, as long as sufficient protein was present initially. To resolve some of these issues, further studies are required to compare the total and allergen-specific IgG responses in dogs receiving different aqueous allergen and alum-precipitated ASIT protocols. It is also important that allergen manufacturers work towards standardisation of allergens so that the precise protein concentrations can be included in vaccines, allowing direct comparison.

A second possibility for the discrepancy may relate to the different methods employed in the various studies. In this study we used a validated semi-quantitative blot analysis system in which the net intensity of bands shows a linear relationship to log antibody concentrations[27] (C. Hou, M.J. Day, T.J. Nuttall, P.B. Hill, unpublished data). This system allows a whole range of separated proteins from *D. farinae* to be studied simultaneously. This is a major advantage compared with ELISA assays, which can only measure a single protein at a time. However, ELISA assays can provide a more accurate measurement of a protein's concentration in mg/ml and they tend to have lower coefficients of variation, indicating superior repeatability. Hence, in future studies, we intend to directly compare the results obtained by blot analysis to ELISA.

Thirdly, the increase in total IgG1 documented in the earlier study[26] was quantified using a radial-immunodiffusion technique. This method measured the total quantity of IgG1 present and did not relate to specific allergens. Hence, the increase seen in that study could have been due to other allergens or non-specific IgG activation and not directly related to *D. farinae* proteins.

Finally, it is possible that production of *D. farinae*-specific IgG antibodies is not induced in dogs with AD undergoing ASIT. This has not previously been studied and it is not known whether the formulation of the allergens, or the expression of the epitopes of major allergens in ASIT, are appropriate or sufficient to stimulate this arm of the immune system. If not, clinical efficacy may be related to some other mechanism such as down-regulation of cellular function or changes in cytokine profiles.

An interesting finding arising from this study was that dogs showing no response to ASIT had significantly higher *D. farinae*-specific total IgG levels on day 0 compared with dogs showing a partial or complete response. This was the case both for total *D. farinae* antigens, and the 98-kDa and 44-kDa proteins. This suggested that if a dog with AD had a pre-existing high level of IgG antibodies to *D. farinae* prior to the onset of ASIT, it was less likely to respond. This is a potentially exciting finding, as it may suggest a means by which dogs could be screened prior to initiation of ASIT. Dogs with a low level of IgG antibodies could be given a better prognosis than those with high levels. Alternatively, this might allow alterations in dosage schedule to be predicted in advance. This finding clearly needs to be substantiated using additional methods and in a larger group of dogs to ensure that it is a repeatable phenomenon. If so, the discriminatory power of the measurement would have to be carefully determined.

A statistical quirk that arose in this study was the finding that dogs receiving the standard-dose ASIT protocol had significantly higher levels of IgG1 to total *D. farinae* proteins at day 0 compared with those receiving the low-dose protocol. This can only have arisen by chance, as the dogs were assigned to their relative groups using block randomisation. This chance occurrence did not seem to have any bearing on the subsequent efficacy of the ASIT because there were no significant differences in response rates between the two groups.[28]

In summary, this study did not demonstrate the production of *D. farinae*-specific IgG blocking antibodies in atopic dogs undergoing ASIT based on alum-precipitated vaccines. However, a high pre-existing level of total IgG to *D. farinae* antigens, including the 98-kDa and 44-kDa proteins, suggested that dogs were less likely to respond to ASIT.

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