



Antibody and Keratinocyte Responses to
***Malassezia pachydermatis* in Dogs**

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DECLARATION

This thesis is of my own composition and the studies described herein were undertaken by myself. The work contained has not been submitted for any other degree or qualification.

Tai-An Chen BVM

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LIST OF ABBREVIATIONS

Ab	Antibody
aFGF	Acidic fibroblast growth factor
Ag	Antigen
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APC	Antigen-presenting cell
APT	Atopy patch test
AR	Amphiregulin
BCA	Bicinchoninic acid
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
B-dNTP	Brominated deoxynucleoside triphosphate
bFGF	Basic fibroblast growth factor
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
BTC	Betacellulin
cAMP	Cyclic adenosine 5'-monophosphate
CAPS	3-(Cyclohexylamino)-1-propanesulfonic acid
CD	Clusters of differentiation
Cdk	Cyclin-dependent kinase
CNBr	Cyanogen bromide
CT	Cholera toxin
DAB	3, 3'-diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum

FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GM-CSF	Granulocyte macrophage-colony stimulating factor
H & E	Haematoxylin & Eosin
HB-EGF	Heparin-binding epidermal growth factor like growth factor
HLA	Human leucocyte antigen
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
IDT	Intradermal test
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IgG-(S-TS)	Short-term sensitising IgG
IL	Interleukin
IRS	Insulin receptor substrate
KGF	Keratinocyte growth factor
MAPK	Mitogen-activated protein kinase
MCP	Membrane cofactor protein
MDDC	Monocyte-derived dendritic cell
mEGF	Murine epidermal growth factor
MEK	MAPK/ERK kinase
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MTS	3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
<i>P</i>	Probability
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PI3K	Phosphatidylinositol-3 kinase

PKB	Protein kinase B
PMS	Phenazine methosulfate
PO	Orally (<i>per os</i>)
PSF	Penicillin, Streptomycin and Fungizone
q	Every
RAST	Radioallergosorbent test
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPT	Skin prick test
SRB	Sulforhodamine B
STAT	Signal transducers and activators of transcription
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TdT	Terminal deoxynucleotidyl transferase enzyme
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TRAF	TNF α receptor associated factor
TSST	Toxic shock syndrome toxin
TTBS	Tris buffered saline containing various concentrations of Tween 20 or Tween 80 (see text for details)
uPA	Urokinase-type plasminogen activator
UV	Ultraviolet
WME	William's Medium E
XTT	Sodium (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide)

ABSTRACT

Malassezia pachydermatis, a lipophilic, unipolar-budding yeast is a commensal on canine skin and it can be isolated from the skin and ear canals of healthy dogs. A dermatitis associated with elevated cutaneous populations of *M. pachydermatis* has been increasingly recognised in the last two decades and one of the most common underlying causes of *Malassezia* overgrowth in dogs is atopic dermatitis. The aim of these studies was to investigate two aspects of the host's protective responses against the organism – activation of a humoral immune response and thickening of the skin by epidermal hyperplasia.

The interaction between *M. pachydermatis* and the host's immune system has not been widely studied either in normal or atopic dogs. To investigate antibody responses to *M. pachydermatis*, Western blotting techniques were used to detect *Malassezia*-specific IgG and IgE binding proteins from *M. pachydermatis* in atopic dogs with or without *Malassezia* dermatitis and normal dogs. By comparing the binding activity on the immunoblots, atopic dogs with *Malassezia* dermatitis showed much greater IgG and IgE responses to *M. pachydermatis* than normal dogs. The IgG in sera from the majority of the atopic dogs with *Malassezia* dermatitis recognised a 25 kDa protein, which was only seen in less than 25 % of the atopic dogs without *Malassezia* dermatitis and none of the normal dogs. Proteins of 45, 52, 56, and 63 kDa were recognised by IgE in more than 50 % of the atopic dogs with *Malassezia* dermatitis, but only recognised by a minority of normal dog sera, and therefore represented major allergens. These results suggest that *Malassezia* dermatitis in atopic dogs is associated with an IgG and IgE response to the organism, with the

above proteins being most relevant in the immunological reaction.

In addition to mounting immunological responses, the skin also undergoes biological changes in response to environmental insults. Epidermal hyperplasia is one of the major characteristics of skin biopsies from dogs with *Malassezia* dermatitis. The mechanisms by which *M. pachydermatis* induces epidermal lesions are not fully understood. To investigate the role of *M. pachydermatis* in the pathogenesis of epidermal hyperplasia associated with *Malassezia* dermatitis, a colourimetric cell proliferation assay was developed to evaluate the effect of extracts and culture supernatants from *M. pachydermatis* on proliferation of canine keratinocytes *in vitro*. *Malassezia* extracts, either with or without protease inhibitors, and culture supernatants from the yeast, did not affect the proliferation of normal canine keratinocytes *in vitro*. To further investigate the response of a direct interaction between canine keratinocytes and live *Malassezia* organisms, an *in vitro* co-culture system was developed. The proliferative response of the keratinocytes was assessed using direct manual counting and immunohistochemistry techniques. *Malassezia* organisms did not cause keratinocyte proliferation, but they induced keratinocyte detachment from the substratum and cell death. These results suggest that the epidermal hyperplasia seen in dogs with *Malassezia* dermatitis is unlikely to be caused by a direct effect of the organism on the keratinocyte cell cycle, but is likely to involve other mechanisms.

These studies provide further information regarding the host response to *M. pachydermatis*, and suggest possible mechanisms by which overgrowth of *Malassezia* organisms causes pathological changes in the skin of dogs.

Chapter 1

GENERAL INTRODUCTION

Malassezia pachydermatis is a lipophilic budding yeast that colonises the skin and mucosal sites of healthy dogs (Guillot & Bond 1999). Despite being part of the normal cutaneous microflora, it has been suggested that the yeast may become a pathogen under some circumstances. As with other *Malassezia* spp. that have been recognised to cause various skin diseases in human beings, *M. pachydermatis* has been associated with cutaneous disease in dogs. “*Malassezia dermatitis*”, a term used to describe inflammation associated with elevated populations of *M. pachydermatis* on the skin of dogs, has been recognised with increasing frequency and its importance in canine dermatology can be illustrated by the rise in the number of review articles on this condition in the last few years (Charach 1997; Bruner & Blakemore 1999; Morris 1999; Bond 2002; Matousek & Campbell 2002).

The interaction between *M. pachydermatis* and canine skin in *Malassezia dermatitis* is not fully understood. Atopic dermatitis is one of the most common underlying causes of *Malassezia* overgrowth in dogs. However, the antigens of the yeast that are recognised by the atopic host thus triggering humoral immune responses have not been reported. Furthermore, the effect of *M. pachydermatis* on epidermal proliferation, one of the characteristic histopathological changes in the skin of dogs with *Malassezia dermatitis*, has not been extensively studied. The deficiencies in the understanding of these important factors of *Malassezia dermatitis* stimulated the studies described in this thesis.

1.1 History and taxonomy of the genus *Malassezia*

The taxonomy and nomenclature of the yeasts currently classified within the genus *Malassezia* have been controversial since the fungus was first described by Eichstedt in 1846 in scales from lesions of pityriasis (tinea) versicolor (cited by Ingham & Cunningham 1993). Due to the presence of filaments associated with the yeasts, the organism was primarily considered as a new species of dermatophyte and designated *Microsporum furfur* by Robin in 1853 (Guého *et al.* 1994). Baillon renamed this filamentous fungus *Malassezia furfur* in 1889 (cited by Assaf & Weil 1996; Ashbee & Evans 2002). On the other hand, Malassez in 1874 described ovoid-shaped cells without hyphae in scales from humans with dandruff and this organism was named *Pityrosporum malassezii* by Sabouraud in 1904 and later referred to as *Pityrosporum ovale* by Castellani and Chambers in 1913 (cited by Sloof 1970). These were initially considered as two different organisms because hyphal forms were rarely observed in dandruff cases and *Pityrosporum* did not produce hyphae in cultures.

The yeast and mycelial forms were later thought to be related and were first classified into the same genus by Panja in 1927 (cited by Ashbee & Evans 2002). In 1951, Gordon (1951) cultured a round-shaped organism from patients with pityriasis versicolor, which he named *Pityrosporum orbiculare*. By using a variety of culture conditions, induction of *P. ovale* and *P. orbiculare* to produce hyphae was demonstrated in the 1970's and the produced hyphae were indistinguishable from those seen in the cases of pityriasis versicolor (Dorn & Roehnert 1977; Nazzaro-Porro *et al.* 1977). This confirmed the dimorphic nature of *Malassezia* organisms and led to the suggestion that round and oval yeast forms and hyphae represented different stages of the life cycle (Salkin & Gordon 1977). The two genera were later

unified with the acceptance of the species name *Malassezia furfur* (including *P. ovale*, *P. orbiculare*, and *M. furfur*) by the International Commission on the Taxonomy of Fungi in 1986 (Cannon 1986). Despite this, many workers continued to differentiate strains on the basis of cellular and colonial morphologies, and the names *P. ovale* and *P. orbiculare* were still widely used in the literature. Due to the heterogeneity of this organism, Midgley (1989) maintained two species, *P. ovale* and *P. orbiculare*, and subdivided the former into three forms (*P. ovale* forms 1-3). Based on the serological differences determined by cell surface antigens, Cunningham *et al.* (1990) designated three serovars A, B, and C within the taxon *M. furfur*.

Malassezia pachydermatis was first identified in 1925 from scales of an Indian rhinoceros with exfoliative dermatitis (Weidman 1925). Due to its resemblance to *P. ovale* but smaller size, Weidman proposed the name *Pityrosporum pachydermatis* for this organism. In 1955, round or oval-shaped yeasts showing unipolar budding were isolated from dogs with otitis externa by Gustafson (1955), and he related them to the genus *Pityrosporum*. In contrast to the lipid dependency of *P. ovale* and *P. orbiculare*, these strains did not require lipid supplementation for growth. Gustafson therefore named his isolates *Pityrosporum canis*. Non-lipid dependent yeasts were also isolated from the ear canals of both healthy dogs and dogs with otitis externa by Fraser (1961) and he termed them *P. pachydermatis* because of the similarity to the isolates described by Weidman (1925). Subsequently, all strains of *Pityrosporum* that were able to grow on media without lipid supplementation were assigned to a single species *P. pachydermatis* by Sloof (1970). After unification of the two genera *Pityrosporum* and *Malassezia* in 1986 (Cannon 1986), the name *Malassezia pachydermatis* was adopted.

Since 1990, the use of genomic analysis has led to a much clearer classification of the yeasts under the genus *Malassezia*. Another lipid-dependent budding yeast *Malassezia sympodialis* was defined by Simmons and Guého in 1990 based on the difference in the percentage of guanine and cytosine in the DNA (% GC) compared to *M. furfur* (Simmons & Guého 1990). Using genomic comparison and ribosomal large subunit sequencing, lipid-dependent *Malassezia* yeasts were further classified into six distinct taxa by Guého *et al.* in 1996, including *M. furfur*, *M. sympodialis*, and four new species, *M. globosa*, *M. obtusa*, *M. restricta*, and *M. slooffiae*, resulting in seven species under the genus *Malassezia* including *M. pachydermatis* (Guého *et al.* 1996). Seven sequence types were also detected within the species *M. pachydermatis* using ribosomal large subunit sequencing (Guillot *et al.* 1997).

In addition to the currently accepted seven *Malassezia* species, two novel species were recently reported to be genetically different from the existing species. Nell *et al.* (2002) isolated *Malassezia* species yeasts from the skin of healthy horses and they proposed the name *M. equi* for this new species that required lipid for growth on Sabouraud's dextrose agar. The other new species was found on the skin of human patients with atopic dermatitis and the name *M. dermatitis* was proposed for the isolates (Sugita *et al.* 2002). The clinical significance of this species is currently unknown.

1.2 Characteristics of *Malassezia* organisms

1.2.1 Structure

A characteristic feature of the genus *Malassezia* is its cell wall structure. The cell wall of *Malassezia* species is relatively thick (up to 0.25 μm) compared to other yeasts and it is multi-layered (Swift & Dunbar 1965; Nishimura *et al.* 1991; Mittag 1995). It constitutes 26 to 37 % of the cell volume (Keddie & Barajas 1972). Around the cell wall there is an outer lamella layer, the structure of which varies depending on the lipid sources in the medium. It is suggested that this lamellar layer contains lipid and may play a role in the adhesion process (Mittag 1995).

The inner surface of the cell wall is corrugated, which corresponds to the invagination of the closely adhered plasma membrane (Barfatani *et al.* 1964; Swift & Dunbar 1965; Mittag 1995). Cytoplasmic organelles that have been described include a nucleus, vacuoles containing lipid, and mitochondria (Barfatani *et al.* 1964; Swift & Dunbar 1965). It was found that the number of mitochondria possessed by round cells (*P. obiculare*) was different from that of oval cells (*P. ovale*). The former contained approximately three mitochondria per cell, which increased in volume rather than number during growth. In contrast, there were approximately 23 mitochondria in an oval cell and they increased in number rather than size during growth (Keddie & Barajas 1972). However, electron microscopic observations did not reveal noticeable differences in the mitochondrial appearance between *P. ovale* and *P. canis* (*M. pachydermatis*) (Swift & Dunbar 1965).

1.2.2 Reproduction

Yeasts of the genus *Malassezia* reproduce by unipolar or sympodial (*M. sympodialis*)

budding and the budding process described for *P. ovale*, *P. orbiculare*, and *M. pachydermatis* is very similar (Swift & Dunbar 1965; Keddie & Barajas 1972; Simmons & Guého 1990; Nishimura *et al.* 1991). However, the budding base of *M. pachydermatis* (0.9-1.1 μm in diameter) is broader than that of *P. ovale* and *P. orbiculare* (0.5-0.7 μm) (Keddie & Barajas 1972; Nishimura *et al.* 1991). Nishimura *et al.* (1991) precisely described the budding process of *M. pachydermatis* in stages. The budding yeast first forms an ear-like structure separating from the cell wall. This structure grows outwards and protrusion of the cytoplasm as well as dimpling of the cell wall takes place. The cell wall of the daughter cell then becomes thicker, layered and serrated as it grows. When the daughter cell is as long as the mother cell, a narrow lumen forms in the cross-wall and fission takes place. The bud is then completely released leaving the ear-like structure as a bud scar.

1.2.3 Biochemistry

Lipid-dependent *Malassezia* species have been reported to elaborate a range of enzymes. Lipase activity has been demonstrated within the cell wall and at the membrane sites of the yeast *in vitro* using histochemical techniques and electron microscopy, suggesting that this enzyme is synthesized intracellularly and exported to the cell surface (Catterall *et al.* 1978). It has also been found that the fatty acid composition of the cell varies depending on the lipid supplementation present in the medium, indicating that fatty acids are required for membrane synthesis (Nazzaro-Porro *et al.* 1976). The lipoxygenase activity of the yeast was proposed to be involved in the pathological changes associated with pityriasis versicolor (Nazzaro-Porro *et al.* 1986; De Luca *et al.* 1996). Increased levels of lipoperoxides were detected in lipids from lesional but not non-lesional skin of patients with pityriasis

versicolor (Nazzaro-Porro *et al.* 1986). The by-products of lipoperoxidation induced by lipoxygenase activity of the yeast were considered to cause damage to melanocytes, resulting in the hypopigmentation seen in pityriasis versicolor (De Luca *et al.* 1996). *In vitro*, *M. furfur* also produces phospholipases (Riciputo *et al.* 1996; Muhsin *et al.* 1997), the activity of which was demonstrated to cause the release of arachidonic acid from human epithelial cell lines, a mechanism by which *Malassezia* organisms may trigger inflammation in the skin (Plotkin *et al.* 1998). In addition to the enzymes described above, *Malassezia* organisms produce azelaic acid in cultures supplemented with oleic acid (Nazzaro-Porro & Passi 1978). Azelaic acid is an inhibitor of tyrosinase, an enzyme involved in the production of melanin, indicating that the production of azelaic acid by *Malassezia* organisms may also play a role in the pathological changes of pityriasis versicolor (Nazzaro-Porro & Passi 1978).

M. pachydermatis has also been demonstrated to produce various enzymes *in vitro*, including alkaline and acid phosphatase, chondroitin-sulphatase, esterase, esterase lipase, galactosidase, glucosidase, hyaluronidase, leucine arylamidase, lipase, lecithinase, peroxidase, phosphoamidase, phospholipase, phosphohydrolase, protease, and urease (Dworecka-Kaszak *et al.* 1994; Kiss *et al.* 1996; Mathieson *et al.* 1998; Guillot & Bond 1999; Coutinho & Paula 2000; Bond 2002). Lipase was found to be predominantly associated with the cell membrane of *M. pachydermatis*, whereas protease is secreted by the yeast (Mathieson *et al.* 1998). No significant difference was found in the production of chondroitin-sulphatase, hyaluronidase, phospholipase, and protease by *M. pachydermatis* strains isolated from dogs with otitis or dermatitis (Coutinho & Paula 2000). When cultured in liquid media, the activities of alkaline phosphatase and esterase lipase in the culture supernatants from *M. pachydermatis*

were significantly greater than that in the cell suspension. Furthermore, significantly greater activity of C4 esterase was detected in cell suspensions of *M. pachydermatis* strains obtained from healthy dogs than that from dogs with dermatitis (Bond 2002).

1.3 *Malassezia* spp. as pathogens in humans and animals

Although *Malassezia* spp. can be found on normal human skin, they have been implicated in a range of both cutaneous and systemic diseases. They are most frequently associated with pityriasis (tinea) versicolor, which is one of the most common disorders of pigmentation seen in human dermatological clinics worldwide. Diseases associated with *Malassezia* spp. in animals have also been widely reported. In dogs, a dermatitis caused by overgrowth of *M. pachydermatis* on the skin surface has been recognised with increasing frequency. The pathogenic role of *Malassezia* spp. in diseases continues to be a major topic in human and veterinary medical literature. In the following sections, the diseases associated with *Malassezia* spp. in humans and animals are reviewed.

1.3.1 Diseases associated with *Malassezia* spp. in man

1.3.1.1 *Pityriasis versicolor*

Pityriasis versicolor is a chronic superficial fungal infection of the skin caused by *Malassezia* spp. organisms (Sunenshine *et al.* 1998; Faergemann 1999). Since the differentiation of new *Malassezia* species, several studies on the mycology of pityriasis versicolor have been documented. The species that have been isolated from patients with pityriasis versicolor include *M. furfur*, *M. globosa*, *M. restricta*, *M. slooffiae*, and *M. sympodialis*, and more than one species can be found in some patients (Erchiga *et al.* 1999; Crespo Erchiga *et al.* 2000; Nakabayashi *et al.* 2000; Gupta *et al.* 2001). Although some investigators suggested that *M. globosa* was the causative agent of pityriasis versicolor due to its predominant presence in populations of affected patients (Crespo Erchiga *et al.* 2000), it remains to be determined whether *M. globosa* is also the predominant species in the lesions of

individuals.

Pityriasis versicolor most often occurs on the trunk, neck and proximal extremities. It is characterised by scaly hypo- or hyperpigmented macules and patches with minimal pruritus (Marcon & Powell 1992). The diagnosis can be made by potassium hydroxide preparations of skin scrapings or tape strippings, which reveal typical clusters of yeast with hyphae (Sunenshine *et al.* 1998; Faergemann 1999). There are several therapeutic options for treating pityriasis versicolor such as topical treatment with lotions or creams containing selenium, sodium thiosulfate or specific anti-fungal agents, or oral medication with ketoconazole, fluconazole or itraconazole. However, relapse is very common and prophylactic treatment may be required (Sunenshine *et al.* 1998).

1.3.1.2 Malassezia folliculitis

Malassezia folliculitis is characterised by follicular papules and pustules localised to the trunk, upper arms, neck, and less often the face. These lesions are generally pruritic (Faergemann 1999). The diagnosis is based on clinical signs, cytology and culture in combination with histopathology. Budding yeasts and rarely hyphae can be found in cytological samples and in dilated follicles of biopsy sections (Assaf & Weil 1996; Faergemann 1999). Although it has been suggested that follicular occlusion was the primary cause of *Malassezia folliculitis* with overgrowth of *Malassezia* organisms as a secondary event (Hill *et al.* 1990), colonisation of normal pilosebaceous units by these yeasts can also be heavy (Leeming *et al.* 1984). The exact role of *Malassezia* organisms in *Malassezia folliculitis* therefore awaits further elucidation. *Malassezia folliculitis* responds rapidly to anti-fungal therapy. It can be

treated with topical anti-fungal agents or with oral azole anti-fungal drugs for patients who do not respond to topical treatment. As with pityriasis versicolor, recurrence tends to be a common problem (Guého *et al.* 1998).

1.3.1.3 *Seborrheic dermatitis and dandruff*

Seborrheic dermatitis is characterised by inflammation and desquamation in areas that are rich in sebaceous glands such as the scalp, face, and upper trunk, whereas dandruff is a non-inflammatory scaling condition of the scalp. It is now generally considered that the latter is the mildest form or a variant of seborrheic dermatitis (Hay & Graham-Brown 1997; Guého *et al.* 1998; Faergemann 1999). The importance of *Malassezia* organisms in these two conditions has been supported by studies demonstrating correlation of in the number of organisms with the severity of the diseases (Heng *et al.* 1990; Pierard-Franchimont *et al.* 1998). The species that have been isolated from patients with seborrheic dermatitis include *M. furfur*, *M. globosa*, and *M. sympodialis* with the first two species showing higher frequency (Nakabayashi *et al.* 2000). Seborrheic dermatitis can be treated with anti-fungal drugs, which can also be used prophylactically to reduce the recurrence rate (Guého *et al.* 1998; Faergemann 1999).

1.3.1.4 *Atopic dermatitis*

Atopic dermatitis is a chronic multi-factorial inflammatory skin disease associated with abnormal immunological regulation and *Malassezia* organisms have been implicated in its pathogenesis as allergens (Ljubojevic *et al.* 2002; Scheynius *et al.* 2002). The IgE response to the organism has been investigated extensively using several diagnostic methods such as the intradermal test, skin prick test, and ELISA

(described in further detail in chapter 4, section 4.1.3.2). Five *Malassezia* species have been isolated from patients with atopic dermatitis, including *M. furfur*, *M. globosa*, *M. restricta*, *M. slooffiae*, and *M. sympodialis*. However, the most frequently isolated species reported varies with different investigators (Nakabayashi *et al.* 2000; Gupta *et al.* 2001; Sugita *et al.* 2001). The respective pathogenic role of different *Malassezia* spp. in atopic dermatitis remains to be clarified. For atopic patients with a hypersensitivity response to *Malassezia* spp., anti-fungal therapy such as oral ketoconazole should be included in the treatment regime (Faergemann 1999).

1.3.1.5 *Malassezia* Fungaemia

Systemic bloodstream infection with *Malassezia* species has been recognised for about two decades. It is related to administration of lipids through intravenous catheters, especially to infants in intensive care units. *M. furfur* and *M. pachydermatis* are the only two species that have been reported to cause systemic disease, and the latter was considered to have been transferred from a household pet since it is rarely isolated from normal human skin (Long & Keyserling 1985; Nicholls *et al.* 1993; Welbel *et al.* 1994; Chang *et al.* 1998). However, other *Malassezia* spp. might also be involved but were not recognised before the new taxonomy was adopted. Many blood culture systems do not effectively support the growth of lipid-dependent *Malassezia* spp. and this may also hinder the identification (Midgley 2000). The catheter has to be removed if anti-fungal therapy is unsuccessful, possibly due to incomplete access to the organisms embedded in the catheter (Ingham & Cunningham 1993; Ashbee & Evans 2002).

In addition to the diseases described above, a range of other conditions have also

been reported to be associated with *Malassezia* spp. such as psoriasis, otitis and acne (listed in Midgley 2000; Ashbee & Evans 2002; Erchiga & Florencio 2002).

1.3.2 Diseases associated with *Malassezia* spp. in other animals

An Indian rhinoceros with exfoliative dermatitis was the first case reported to be associated with *M. pachydermatis* (Weidman 1925). More recently, similar lesions were observed in a southern white rhinoceros (Bauwens *et al.* 1996). *Malassezia* organisms have also been found in a variety of other animals with diseases. Non-lipid dependent *Malassezia* yeasts were recently isolated from a horse showing an erythematous patch of alopecia on the face, which responded well to a miconazole/chlorhexidine shampoo (Paterson 2002). *M. pachydermatis*-associated dermatitis has also been reported in sea lions (Guillot *et al.* 1998; Nakagaki *et al.* 2000). Other than dogs and cats, otitis externa caused by *M. pachydermatis* has been described in ferrets, fennecs, pigs, and dromedaries (Dinsdale & Rest 1995; Guillot & Bond 1999). In contrast, the species that have been isolated from cattle with otitis are *M. globosa*, *M. sympodialis*, *M. furfur*, and *M. slooffiae*, with a higher frequency of the first two species (Duarte *et al.* 1999; Duarte *et al.* 2001). A case of non-cutaneous disease was documented in a bird (macaw) showing an ulcer in the crop from which *M. pachydermatis* was isolated (Breuer-Strosberg *et al.* 1990).

In contrast to dogs, in which *Malassezia* dermatitis and otitis are frequently diagnosed, these conditions are less common in cats. The clinical signs in cats include pruritus, erythema, self-excoriation, and less commonly lichenification (Morris 1999). *M. pachydermatis* has also been reported to cause feline chin acne (White *et al.* 1997). Although three lipid-dependent *Malassezia* spp. (*M. furfur*, *M.*

globosa, and *M. sympodialis*) have been isolated from healthy cats (Bond *et al.* 1996a; Bond *et al.* 1997; Bensignor *et al.* 1999; Crespo *et al.* 1999), to date only *M. sympodialis* has been reported to be associated with otitis externa in cats (Crespo *et al.* 2000; Crespo *et al.* 2002). *Malassezia* overgrowth has also been reported in cats with thymoma-associated dermatitis and paraneoplastic alopecia, suggesting a correlation between *Malassezia* dermatitis and internal neoplasia in cats (Forster-Van Hijfte *et al.* 1997; Godfrey 1998; Mauldin *et al.* 2002).

1.3.3 *Malassezia* dermatitis in dogs

Malassezia dermatitis in dogs was first reported by Dufait (1983) 20 years ago. It is associated with elevated cutaneous populations of *M. pachydermatis*. To date, the potentially important role of *M. pachydermatis* in this condition has gained widespread acceptance due to the recovery of *M. pachydermatis* from affected skin of dogs with more generalised skin diseases and the favourable outcome after anti-fungal therapy. The following sections will review the current knowledge of *Malassezia* dermatitis associated with *M. pachydermatis* in dogs, with emphasis on its predisposing factors, pathogenesis and clinical aspects.

1.3.3.1 Predisposing factors for overgrowth of *Malassezia pachydermatis*

The predisposing factors for *Malassezia* overgrowth on the skin of dogs are still a focus of research and debate. Two mechanisms that have been suggested to trigger overgrowth of the yeast are changes in the cutaneous microenvironment and alterations in host defense mechanisms (Mason 1993; Matousek & Campbell 2002). By causing these changes, various diseases have been reported as underlying causes of *Malassezia* dermatitis.

The cutaneous microenvironment is generally considered to be important in controlling *Malassezia* populations. *Malassezia* dermatitis seems to be more common in humid climates such as summer and in certain anatomic sites such as skin folds, suggesting that increased cutaneous humidity favours yeast growth (Scott *et al.* 2001a). Additionally, changes in lipids on the skin surface resulting in increased availability of nutrients and growth factors for *Malassezia* organisms can also promote their proliferation. The diseases that can cause changes in sebum production and are associated with *Malassezia* dermatitis include endocrine diseases, bacterial skin diseases (in which the bacteria could release lipase), and keratinization disorders such as seborrheic dermatitis (Plant *et al.* 1992; Scott *et al.* 2001a; Matousek & Campbell 2002).

The skin forms a physical barrier to resist environmental insults such as invasion by microorganisms. A disrupted epidermal barrier renders the skin prone to bacterial and yeast infections. Diseases that can cause a decrease in cutaneous barrier function and are commonly related to *Malassezia* dermatitis are hypersensitivity diseases (especially atopic dermatitis), parasite infestation, and keratinization disorders (Scott *et al.* 2001a; Matousek & Campbell 2002). Another defense mechanism of the host is the skin immune system. Alterations in the immune system caused by hypersensitivity and endocrine diseases can also predispose to *Malassezia* dermatitis (Matousek & Campbell 2002).

Genetic predisposition appears to be important in certain breeds, especially West Highland white terriers, Basset hounds, Dachshunds, Cocker spaniels, Shih Tzus, and English setters (Plant *et al.* 1992; Bond *et al.* 1996b; Scott *et al.* 2001a). Furthermore,

medications such as long-term glucocorticoid therapy may also be predisposing factors (Scott *et al.* 2001a). However, some investigators suggested that antibiotic treatment was associated with increased *Malassezia* populations (Plant *et al.* 1992), whereas others did not support this point of view (Matousek & Campbell 2002).

1.3.3.2 Pathogenesis

The pathogenesis of *Malassezia* dermatitis in dogs has not been fully elucidated. Several mechanisms by which *M. pachydermatis* might cause pathological changes in the skin of dogs have been proposed. Zymogen in the yeast cell wall is capable of activating the complement system. This can result in damage to epidermal integrity, which is considered to cause epidermal spongiosis, inflammation and pruritus. The damaged epidermal water barrier can also lead to an increase in humidity on the skin surface thus favouring yeast proliferation (Mason 1993). As proteases are believed to be the mediator of itch at free nerve endings in the skin, the proteases released by *Malassezia* organisms may also contribute to the pruritus (Mason & Evans 1991). *Malassezia* organisms produce lipases, which alter sebum production and produce free fatty acids on the skin surface. Released lipid can be used by the yeast for nutrition, and free fatty acids provide protection by inhibiting other organisms (Mason *et al.* 1996). T cell-mediated immunity is important in prevention and recovery from fungal infections. A deficient cell-mediated defense system may predispose the host to overgrowth of *Malassezia* organisms (Mason 1993). Additionally, the disrupted epidermal barrier could permit the skin immune system to be exposed to *Malassezia* antigens and products, eliciting inflammatory and/or hypersensitivity reactions (Scott *et al.* 2001a).

1.3.3.3 Clinical features of Malassezia dermatitis

1.3.3.3.1 Clinical presentation

Malassezia dermatitis occurs in dogs of any age, sex, and breed, but is more often diagnosed in dogs between one and three years of age (Mason & Stewart 1993). Some breeds also appear to be predisposed (listed in section 1.3.3.1). The dermatitis often begins in the summer or highly humid months, which also corresponds to the allergy season, and then persists into winter. There is a second spike of cases in early spring (Mason 1993; Scott *et al.* 2001a).

Skin lesions may be localised or generalised. Regional dermatitis commonly occurs in the external ear canal, or on the face, ventral neck, axillae, groin, interdigital skin or intertriginous areas. Skin lesions are characterised by erythema, alopecia, greasy exudation and varying degrees of scaling. Chronic cases can have marked hyperpigmentation and lichenification. Pruritus varies from mild to extremely severe. Dogs with generalised lesions often have an offensive, rancid or yeasty odour. *Malassezia* paronychia may occur with or without more generalised *Malassezia* dermatitis. It produces a reddish-brown staining of hairs or claws, with inflammation of the surrounding soft tissue. Most dogs with *Malassezia* dermatitis have concurrent dermatoses, especially hypersensitivity disorders, ectoparasitic infestation, bacterial pyoderma, endocrinopathies, and keratinisation defects (Mason 1993; Mason & Stewart 1993; Bond 1997; Morris 1999; Scott *et al.* 2001a).

1.3.3.3.2 Diagnosis

The criteria required for the diagnosis of *Malassezia* dermatitis have not been firmly established. It has been proposed that a diagnosis of “*Malassezia* dermatitis” is

appropriate when a dog with elevated *M. pachydermatis* populations on lesional skin shows a good clinical and mycological response to appropriate anti-fungal therapy (Bond & Lloyd 1997). The diagnostic tools used to identify *Malassezia* yeasts on the skin include cytological, cultural, and histopathological techniques.

Cytology

Cytological examination is a useful technique allowing *Malassezia* populations to be rapidly assessed. A variety of methods have been used to collect cytological samples for the evaluation of the organisms, including vigorously rubbing a cotton swab on the skin surface, pressing a piece of clear acetate tape onto lesional skin several times, making a superficial skin scraping, and directly impressing a glass slide onto lesional skin (Mason 1993; Scott *et al.* 2001a). Cotton swabs and tape stripping are useful for interdigital spaces. Tape stripping and skin scraping may be more effective for drier areas. Direct impression with a glass slide is possible for flat surfaces with grease and wax (Morris 1999; Scott *et al.* 2001a; Matousek & Campbell 2002). It is difficult to determine which sampling method is the best since each has its own benefits and disadvantages. It has been found that swabs, skin scrapings and impression smears gave similar results in normal dogs (Kennis *et al.* 1996). Using dogs with elevated cutaneous *Malassezia* populations as sampling subjects, Bond and Sant (1993) observed relatively higher numbers of *Malassezia* organisms on materials prepared by tape stripping and dry scraping than by damp swabs. Some investigators reported that direct impression with a glass slide was the most reliable technique for producing uniform cytological preparations (Plant *et al.* 1992).

With the exception of tape strips, the samples collected are transferred to a glass slide,

heat-fixed, and stained with Diff-Quik[®], Giemsa, Gram's or methylene blue. The tape is dipped into Diff-Quik[®] solution II or stained with methylene blue without heat fixation and applied to a glass slide with the sticky side down (Matousek & Campbell 2002). The slides are examined under high power or with an oil immersion lens on a light microscope. Microscopic examination reveals round to oval yeasts (3 to 8 μm in diameter) with monopolar budding (Figure 1.1). Yeasts are often seen in clusters or adhered to keratinocytes (Scott *et al.* 2001a). As *M. pachydermatis* can be found in a small number on healthy dog skin, it is difficult to define the amount of yeast that is pathogenic. It has been suggested that populations should be considered elevated if the yeast is readily identified (Bond 1997). Some investigators also suggested that an elevated population is more likely when a certain number of yeast is found (Scott *et al.* 2001a). The various criteria proposed include greater than 10 organisms in 15 randomly chosen oil-immersion microscopic fields ($\times 1000$) using tape stripping samples, an average of greater than or equal to four organisms per oil-immersion microscopic field, an average of greater than or equal to one organism in 10 oil-immersion microscopic fields, and greater than two organisms per high power field ($\times 400$) with specimens obtained using any of the commonly used sampling techniques.

Culture

Four methods have been described to culture *Malassezia* organisms from the skin, including cotton swabs (Bond *et al.* 1995a), adhesive tapes (Noble & Somerville 1974; Kennis *et al.* 1996), contact plates (Bond *et al.* 1994a), and detergent scrubs (Bond *et al.* 1995b). *M. pachydermatis* grows well on both Sabouraud's dextrose agar and modified Dixon's agar at 32°C to 37°C. However, an atmosphere

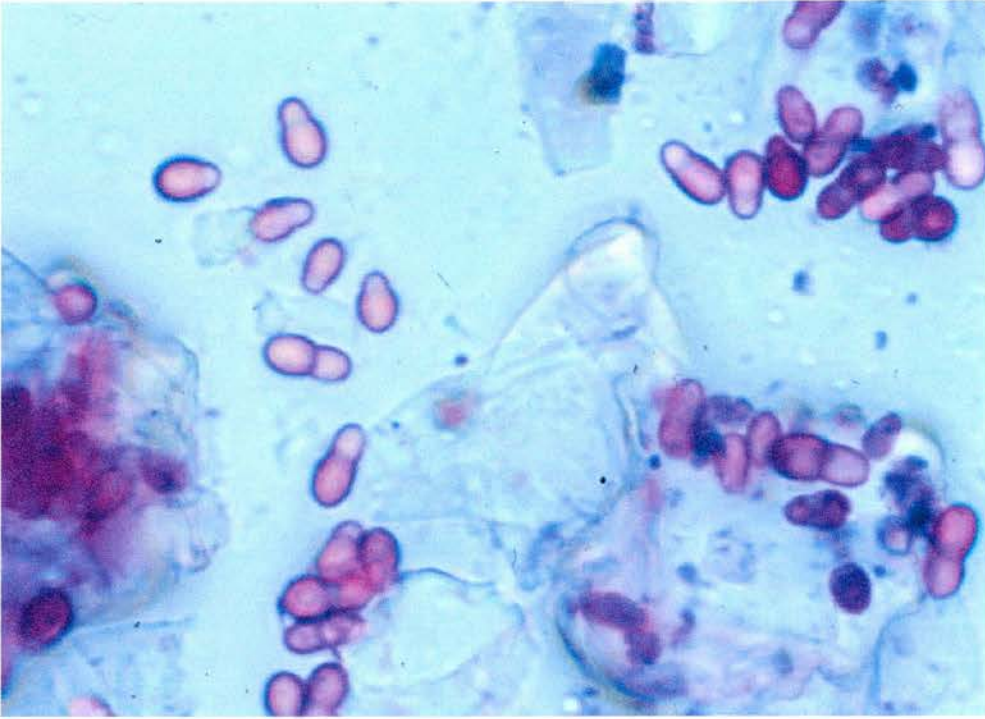


Figure 1.1 Cytological examination of a Diff-Quik[®] stained tape strip obtained from the skin of a dog with *Malassezia* dermatitis (x1000)

Numerous round to oval or peanut-shaped budding yeasts are present.

containing 5 to 10 percent carbon dioxide significantly increases the frequency of isolation and colony counts on Sabouraud's dextrose agar, but not on modified Dixon's agar (Bond & Lloyd 1996a). Nevertheless, modified Dixon's agar, a lipid-supplemented medium, may be advantageous for diagnostic purposes because it supports the growth of more lipid-dependent variants of *M. pachydermatis* (Bond & Anthony 1995) and the lipid-dependent *Malassezia* spp. that may be found on cats (Bond *et al.* 1996a; Bond *et al.* 1997; Bensignor *et al.* 1999; Crespo *et al.* 1999). Since *Malassezia* spp. are commensal organisms and elevated populations can be readily found on cytology, culturing is not usually necessary and is of little practical diagnostic value (Scott *et al.* 2001a; Matousek & Campbell 2002).

Histopathology

Malassezia organisms may also be demonstrated by microscopic examination of skin biopsy specimens (Figure 1.2 A-C). The yeasts are located in the stratum corneum (Figure 1.2 C) and are occasionally seen in the follicular infundibulum. Due to the possible loss or disruption of the stratum corneum during processing, skin biopsy is generally considered to be less sensitive and reliable than other diagnostic tools. There are several characteristics in skin biopsy samples from dogs with *Malassezia* dermatitis (Scott & Miller 1989; Yager 1994; Mauldin *et al.* 1997; Muse 2000; Nett *et al.* 2001; Scott *et al.* 2001a). The epidermis is characterised by marked irregular hyperplasia with formation of deep rete ridges (Figure 1.2 A). Hyperplasia can also be seen in the follicular infundibula. Orthokeratotic hyperkeratosis with focal parakeratosis is frequently observed. Spongiosis is marked in active lesions (Figure 1.2 B). The dermal inflammation is usually superficial perivascular to interstitial with lymphocyte exocytosis and focal accumulations of neutrophils. Eosinophils are

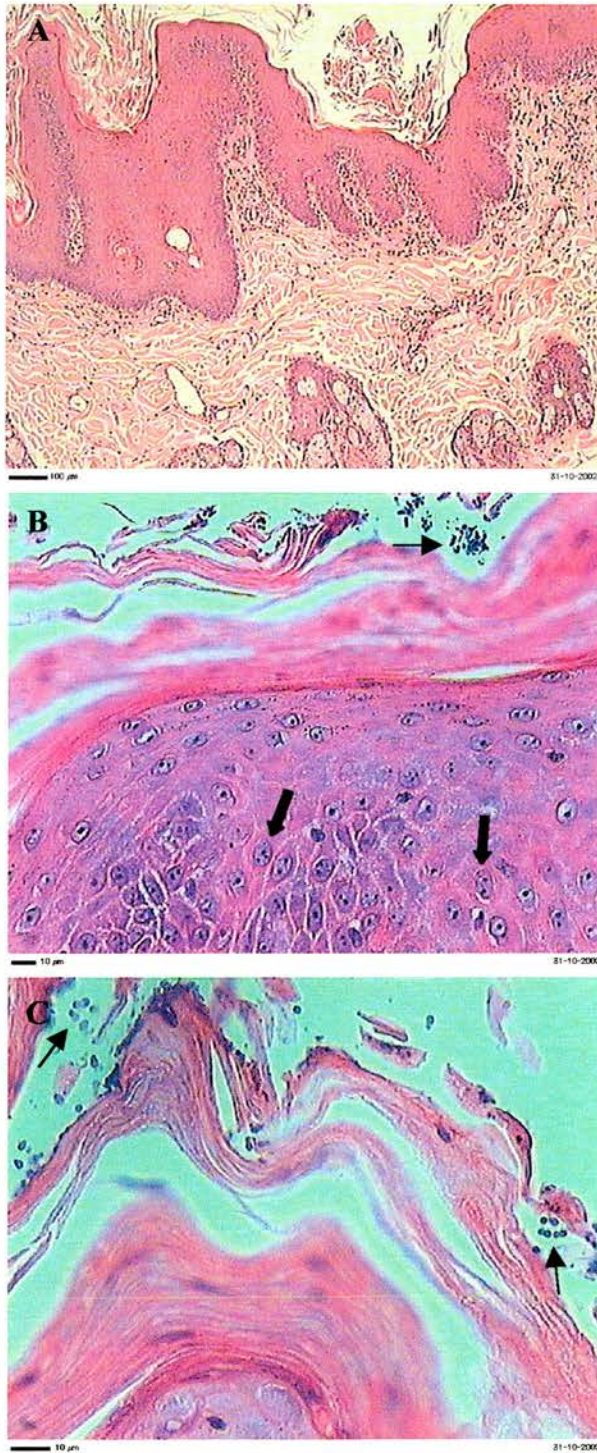


Figure 1.2 A-C Histopathology of canine *Malassezia* dermatitis (x40, x250, x400)

A: Irregular epidermal hyperplasia and hyperkeratosis. B: The epidermis shows marked spongiosis and orthokeratotic hyperkeratosis with focal parakeratosis. *Malassezia* yeasts are visible in the stratum corneum (thin arrow). Some keratinocytes show multiple nucleoli, indicating mitotic activity (bold arrows). C: Budding yeasts showing characteristic morphology of *Malassezia* spp. (thin arrows). Scale bars for A, B, and C are 100, 10, and 10 μ m, respectively. The glass slide was kindly provided by Dr. S. Rhind, University of Edinburgh.

present occasionally. Linear alignment of mast cells at the dermoepidermal junction can also be observed in some affected dogs (Mauldin *et al.* 1997; Beningo *et al.* 2000). Scott and Miller (1989) described a hyperplastic dermatosis associated with secondary *M. pachydermatis* infection in West Highland White Terriers. The epidermal hyperplasia in these dogs took the form of round bottomed rete ridges, which the investigators described as epidermal dysplasia.

1.3.3.3.3 Treatment

The treatment of *Malassezia* dermatitis is currently based on topical and systemic anti-fungal therapy (Morris 1999). A combination of topical and systemic therapy can speed resolution of the disease and increase efficacy (Scott *et al.* 2001a; Matousek & Campbell 2002).

Topical agents that can be used for *Malassezia* dermatitis include chlorhexidine, clotrimazole, enilconazole, ketoconazole, miconazole, nystatin and selenium sulfide (Mason 1993; Scott *et al.* 2001a). It has been reported that *M. pachydermatis* showed sensitivity, in decreasing order of efficacy, to ketoconazole, econazole, clotrimazole, miconazole, and nystatin *in vitro* (Kiss *et al.* 1997). These agents are used in various forms of topical anti-fungal products such as sprays, ointments, and shampoos. A double-blind study demonstrated that a shampoo containing 2 % chlorhexidine and 2 % miconazole (Malaseb[®], Leo Animal Health) when used every three days for three weeks was effective for treating *Malassezia* dermatitis in dogs due to its degreasing, anti-*Malassezia*, and anti-bacterial properties (Bond *et al.* 1995c).

The commonly used systemic agents for *Malassezia* dermatitis in dogs are the azoles.

Ketoconazole or itraconazole are usually given at 5-10 mg/kg/day PO for 21-30 days (Morris 1999; Matousek & Campbell 2002). However, adverse effects including anorexia, vomiting, diarrhoea, and hepatotoxicity have been reported (Morris 1999). A recent study demonstrated that itraconazole given at 5 mg/kg q 24h PO on 2 consecutive days per week for 3 weeks (pulse administration) was as effective as when given at 5 mg/kg q 24h PO daily for 21 days in treating canine *Malassezia* dermatitis (Pinchbeck *et al.* 2002).

Clinical improvement usually occurs within 7 to 14 days after initiation of anti-fungal therapy. As *Malassezia* dermatitis in dogs is usually associated with an ongoing disorder, the dog should be evaluated for concurrent diseases. For frequently recurring cases, shampoo therapy or pulse oral medication may be used prophylactically (Bond 1997; Morris 1999; Scott *et al.* 2001a).

1.4 Cutaneous responses to *Malassezia* organisms

The skin is continually challenged by a variety of environmental hazards. The first line of defense and protection is provided by the epidermis, the outermost skin layer (Eckert *et al.* 1997). The epidermis protects the body from water loss, UV damage, mechanical injury, microbiological invasion, and immunological insults. The predominant cell type of the epidermis, the keratinocyte, provides mechanical protection from parasites and microorganisms, and it also forms the first line of immunological defense against environmental or microbiological antigens and allergens (Blumenberg & Tomic-Canic 1997).

The skin tends to respond to environmental insults by activating the skin immune system and increasing the thickness of the skin. The latter results from increased proliferation of cells in the basal layer of the epidermis, leading to epidermal hyperplasia, hyperkeratosis and lichenification as seen clinically. Inflammation and lichenification are generally seen in the skin of dogs with *Malassezia* dermatitis (section 1.3.3.3.1), indicating that both protective mechanisms are involved in the disease process. Aspects of these two mechanisms were investigated in the studies described in chapters 3-6, and the two mechanisms are discussed in detail in the following two sections.

1.4.1 The immune response to *Malassezia* organisms

The skin immune system consists of cells residing in the skin, including keratinocytes, Langerhans' cells, dermal dendrocytes, and mast cells, as well as skin-seeking T lymphocytes and endothelial cells of cutaneous post-capillary venules (Yager 1993). It functions in conjunction with humoral substances, such as secretory

immunoglobulins and cytokines, to deal with challenges from both exogenous and endogenous antigens, to mount effector responses of various types, and to balance the response to minimise tissue damage while eliminating the original insult (Yager 1993). Defects in both innate and acquired immune responses may lead to the development of skin diseases. In sections 1.4.1.1 and 1.4.1.2, the literature regarding the immunological responses to *Malassezia* organisms in both humans and dogs are reviewed.

1.4.1.1 Non-allergic immunological responses to Malassezia organisms

Immunoglobulins specific to the yeast phase of *Malassezia* organisms have been studied in normal human individuals with no history of skin disease at various age ranges. Sohnle *et al.* (1983) measured IgM, IgG and IgA antibodies against *Malassezia* organisms by ELISA in sera from 21 young subjects (aged 23-44) and 20 elderly subjects (aged 70-88), none of whom had a history of “significant” superficial fungal infections. It was found that all three classes of immunoglobulins to *Malassezia* organisms were present in both the young and elderly groups, and the responses of the two groups were very similar, except for the IgM response. The level of *Malassezia*-specific IgM in the elderly group was significantly lower than that in the young subjects. In another study investigating *Malassezia*-specific IgG in the sera of normal individuals aged from 29 to 81, the antibody titers were shown to decline with increasing age, which paralleled the decreasing populations of *Malassezia* organisms detected on the skin of elderly people (Bergbrant & Faergemann 1988). Lower titers of *Malassezia*-specific IgG have also been reported in healthy children. Substantially lower levels of *Malassezia*-specific IgG were detected in sera from five healthy 6 month-old children compared to 10 healthy

adults, but no statistical analysis was performed on the results in this study (Johansson & Faergemann 1990). Cunningham *et al.* (1992) studied IgM, IgG and IgA responses to *M. furfur* serovar A, B and C in normal individuals divided into 5 age groups, including 2-3 years, 7-10 years, 20-24 years, 33-40 years, and 60-64 years. There was no significant difference in the titers of the three immunoglobulins between age groups as measured by ELISA, except that the subjects aged between 60 and 64 had significantly lower *Malassezia*-specific IgM titers than the others. The *Malassezia*-specific IgA level was found to be low in all groups, suggesting that mucosal sensitisation by the yeast might not be important. Due to the difficulties in producing the mycelial phase of *Malassezia* organisms *in vitro*, very few investigators have studied the humoral responses to the mycelium of the yeast. One study reported that healthy adults had detectable IgM, IgG, IgG subclasses and IgA to mycelial antigens of *Malassezia* organisms in their sera, with the highest titers being found for IgG (cited in Ashbee & Evans 2002).

Considerably fewer studies have been carried out on cell-mediated immunity to *Malassezia* organisms in healthy human individuals at various ages. In order to gain a clearer picture of cell-mediated immunity to the yeast, Ashbee and Evans (2002) collated data from control subjects in various studies ranging from 8 to 61 years of age. They concluded that *Malassezia* organisms could elicit significant cell-mediated immune responses in healthy individuals as measured by lymphocyte transformation assays or a leucocyte migration inhibition assay. Also, the responses were similar in different age groups, suggesting that the level of cell-mediated immunity to *Malassezia* organisms remains fairly constant throughout life. One recent study looking at cell-mediated immunity to the mycelial phase of *Malassezia* organisms

reported that positive responses were detected in 75 % of the healthy individuals studied using a leucocyte migration inhibition assay (Saadatzadeh *et al.* 2001).

Bond *et al.* (1998) studied the humoral and cell-mediated immune responses to *M. pachydermatis* in healthy dogs and dogs with *Malassezia* dermatitis using ELISA and a lymphocyte proliferation assay. Serum titers of *Malassezia*-specific IgG and IgA in seborrheic Basset hounds with high cutaneous populations of *M. pachydermatis* and affected dogs of various breeds were found to be significantly greater than those of healthy Basset hounds and healthy Beagles. The investigators concluded that high serum titers of IgG and IgA do not prevent seborrheic dermatitis associated with *M. pachydermatis* in either Basset hounds or other breeds. The peripheral blood mononuclear cell (PBMC) proliferative responses to *M. pachydermatis* antigens at a concentration of 500 µg/ml in seborrheic Basset hounds and Irish setters with gluten-sensitive enteropathy were significantly lower than those in healthy Basset hounds. There was no significant difference between the PBMC responses of the first two groups and healthy Beagles. The responses to *Malassezia* antigens at 50 µg/ml in the four groups of dogs, however, did not vary significantly. The role of reduced PBMC responses to *M. pachydermatis* in the pathogenesis of *Malassezia* dermatitis in seborrheic Basset hounds is yet unclear.

1.4.1.2 Immunological responses to Malassezia organisms in atopic dermatitis

Elevated cutaneous populations of *Malassezia* organisms have been associated with atopic dermatitis both in man and dogs. The immune response to the yeast in human patients with atopic dermatitis has been studied extensively, and their role as an allergen in some atopic patients has been reviewed (Faergemann 1999; Scheynius *et*

al. 2002). In contrast, relatively few studies have been documented in dogs. Based on information from the existing literature, a hypothesis to explain the mechanisms by which overgrowth of *M. pachydermatis* might cause hypersensitivity reactions in dogs with atopic dermatitis can be formulated (summarised in Figure 1.3). It is postulated that *M. pachydermatis* on the skin surface releases antigens that penetrate the skin and are captured by epidermal Langerhans' cells or dermal dendritic antigen-presenting cells. These cells would then migrate to regional lymph nodes and present the antigen to a T lymphocyte through a major histocompatibility complex (MHC) class II molecule, which in cooperation with different environmental cytokines stimulates T helper (Th) 0 precursor cells to differentiate into Th1 cells or Th2 cells. A cytokine environment dominated by IL-12 favours Th1 cell development, whereas IL-4 and IL-13 stimulate the development of Th2 cells. Th cells would activate B lymphocytes and stimulate them to differentiate into antibody-forming plasma cells. By secreting IL-2 and IFN- γ , Th1 cells promote IgG production, whereas Th2 cells produce IL-4 and IL-13 that promote immunoglobulin class switching to IgE. The role of *Malassezia*-specific IgG antibodies in atopic dogs with *Malassezia* dermatitis is currently unclear. The IgG antibodies might provide some protective immunity against *Malassezia* organisms. Alternatively, they might activate the complement system causing epidermal damage and inflammation. The allergen-specific IgE antibodies would bind to the receptors on the surface of mast cells and thus sensitise them. Subsequent exposure to *Malassezia* allergens would cross-link surface-bound IgE and trigger the release of a variety of inflammatory mediators from mast cells, resulting in clinical symptoms of Type I hypersensitivity reactions (Figure 1.3). The evidence in the literature that supports this proposed pathogenetic pathway is reviewed in the following sections.

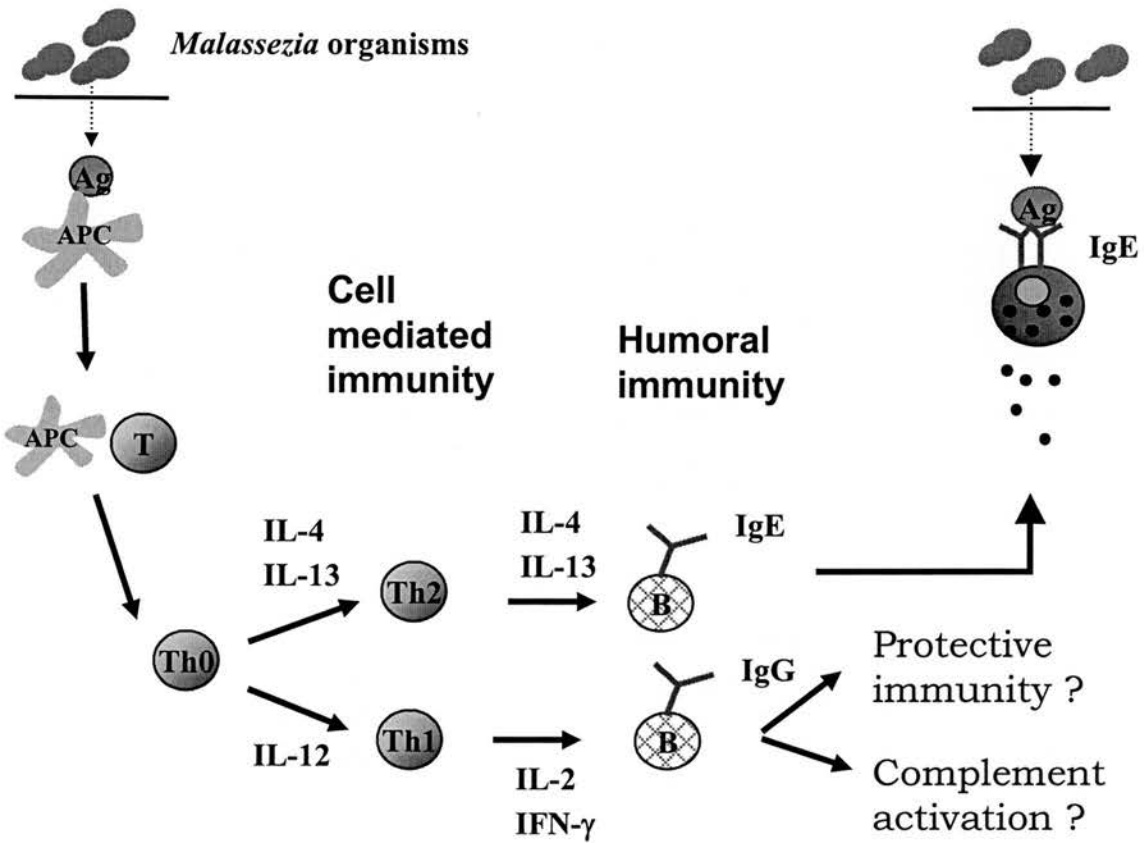


Figure 1.3 Possible pathways for immunological responses stimulated by *Malassezia* organisms in atopic dermatitis

Malassezia organisms on the skin surface might release antigens (Ag) that penetrate the skin and be captured by an antigen-presenting cell (APC). The APC would then present the antigens to a T lymphocyte. The cytokines present in the environment determines which T cell subset is produced from Th0 cells. IL-12 stimulates development of T helper 1 (Th1) cells. IL-4 and IL-13 favour the production of T helper 2 (Th2) cells. Th1 cells secrete IL-2 and IFN- γ that induce IgG responses. *Malassezia*-specific IgG might be protective or activate complement provoking inflammation. Th2 cells secrete IL-4 and IL-13 that induce allergen-specific IgE responses. The IgE antibodies bind to mast cells and cross-link upon subsequent encounter with allergens, which triggers mast degranulation and causes clinical features of Type I hypersensitivity reactions.

1.4.1.2.1 Antigen release, penetration, and presentation

It has been suggested that entry through the skin is the most likely mechanism by which *Malassezia* organisms stimulate the immune system in human patients with atopic dermatitis (Scheynius *et al.* 2002). A recent study demonstrated that *M. furfur* was able to invade human keratinocytes and resist phagolysosome fusion (Baroni *et al.* 2001a). It has also been reported that the first cloned *Malassezia* allergen (Mal s 1), a major allergen that is localised to the cell wall, can be released into the culture medium (Scheynius *et al.* 2002). These findings support the hypothesis that *Malassezia* organisms can penetrate the epidermal barrier and probably release allergens in the skin, where both whole organisms and allergenic components would come into contact with Langerhans' cells in the epidermis. Uptake of whole *M. furfur* yeast cells and various allergenic components from the yeast, including *M. furfur* extracts, rMal f 5 and *M. furfur* mannan, has been demonstrated *in vitro* using immature monocyte-derived dendritic cells (MDDCs) which reflect Langerhans' cells in the skin (Buentke *et al.* 2000). These results suggest that sensitisation of atopic patients to *M. furfur* can be mediated by immature dendritic cells in the absence of IgE in the skin. The internalisation was shown to occur via binding to the mannose receptor (other receptors may also be involved) or pinocytosis (Buentke *et al.* 2000). The presence of *M. furfur* was also shown to induce maturation of immature MDDCs by up-regulation of CD83 expression, and increase in expression of co-stimulatory molecules, CD80 and CD86 (Buentke *et al.* 2001). The mature dendritic cells are poor at antigen uptake, but excellent at presenting antigens. They would then efficiently present antigen-derived peptides on MHC molecules to T cells (Banchereau & Steinman 1998).

There has been some evidence implying that the interaction between *Malassezia* antigen-bearing antigen-presenting cells (APCs) and T cells takes place in the skin. It has been observed that in *P. orbiculare* patch test-positive atopic dermatitis patients, an infiltration of CD4⁺ T cells was detected at 24 hours at the patch test sites, but was more pronounced at 72 hours (Tengvall Linder *et al.* 2000). The expression of intercellular adhesion molecules (ICAM)-1 and human leucocyte antigen (HLA)-DR in the dermis of these patients was also up-regulated. At 24 hours post-test, the former correlated with the scale of the dermal CD3⁺ infiltrates, with the majority being CD4⁺ (Tengvall Linder *et al.* 2000).

To date, the release, penetration and presentation of *Malassezia* antigens into/in the skin of dogs has not been studied. However, there are some data providing indirect evidence for a transepidermal route of antigen penetration. Higher numbers of Langerhans' cells have been detected in lesional atopic skin compared to clinically normal atopic or normal control canine skin (Day 1996; Olivry *et al.* 1996). These cells were present in clusters in lesional skin of dogs with atopic dermatitis (Olivry & Hill 2001). Expression of surface IgE was also observed on epidermal Langerhans' cells in lesional atopic canine skin (Olivry *et al.* 1996), and those cells are responsible for allergen capture and presentation (Olivry & Hill 2001). Moreover, eosinophils can be seen below the stratum corneum in lesional atopic canine skin, but not in clinically normal atopic skin (Olivry *et al.* 1997). Canine atopic skin also exhibits hyperplasia of T lymphocytes expressing the gamma-delta T-cell receptor (Olivry *et al.* 1997). Furthermore, transepidermal penetration of staphylococcus antigens has been demonstrated in dogs (Mason & Lloyd 1989). Taken together, these findings indicate that it is likely that *M. pachydermatis* may be able to release

antigens that would penetrate the skin of atopic dogs where they are captured by epidermal APCs, Langerhans' cells, and thus initiate the process of antigen presentation to T cells and a cascade of immunological responses.

1.4.1.2.2 Cell-mediated immune responses

Cell-mediated immune responses to *Malassezia* organisms have been investigated both in man and dogs using lymphocyte proliferation assays. In humans, it has been shown that immature MDDCs pre-incubated with *M. furfur* induced a proliferative response in autologous PBMCs in a dose-dependent manner (Buentke *et al.* 2001). The proliferative responses of PBMCs to *Malassezia* organisms (*P. orbiculare* or *P. ovale*) in human patients with atopic dermatitis have been demonstrated to be significantly higher than that in healthy individuals in several studies (Rokugo *et al.* 1990; Tengvall Linder *et al.* 1996; Tengvall Linder *et al.* 1998; Savolainen *et al.* 2001). Furthermore, atopic patients with specific IgE antibodies against *P. orbiculare* or *P. ovale* were shown to have increased synthesis of the Th2-related cytokines, IL-4, IL-5, and IL-10, by *Pityrosporum*-stimulated PBMCs (Kröger *et al.* 1995; Tengvall Linder *et al.* 1998). The results of several studies investigating the correlation between PBMC proliferation and skin test responses to *Malassezia* antigens are inconsistent. Some investigators reported that a correlation was not found between the PBMC proliferation and the skin prick test (SPT) or atopy patch test (APT) in atopic dermatitis patients (Rokugo *et al.* 1990; Tengvall Linder *et al.* 2000). In contrast, Johansson *et al.* (2002) showed a correlation between PBMC proliferation in response to *in vitro* stimulation with *Malassezia* extracts and *M. furfur* APT reactions in atopic patients with positive SPT reactions to the yeast. Additionally, both PBMC proliferation and APT reactions to *Malassezia* extracts were also found

to correlate with the production of Th2-related cytokines, IL-4, IL-5, and IL-13, by PBMCs in these patients (Johansson *et al.* 2002). In one study investigating T lymphocyte clones isolated from lesional atopic skin, the majority of the T cells that were reactive for *P. orbiculare* showed a Th2 or Th2/Th0 like cytokine profile (Tengvall Linder *et al.* 1996). By examining T cell receptor usage to determine whether *Malassezia* organisms can act as superantigens, no evidence has been found for superantigenic activity by *P. orbiculare* in patients with atopic dermatitis (Johansson *et al.* 1999).

The PBMC response to *M. pachydermatis* in atopic dogs has been studied recently. Atopic dogs with cytological evidence of *Malassezia* overgrowth had significantly higher PBMC responses to extracts of *M. pachydermatis* than clinically normal dogs. Atopic dogs without *Malassezia* overgrowth had PBMC responses that fell between those of the above two groups (Morris *et al.* 2002). A significant correlation was not found between the PBMC proliferation and intradermal test response to *M. pachydermatis* in atopic dogs in this study (Morris *et al.* 2002).

1.4.1.2.3 Humoral immune responses

Using *in vitro* serological tests such as ELISA and the radioallergosorbent test (RAST), *Malassezia*-specific IgE has been detected in human atopic patients for over a decade. Kröger *et al.* (1995) studied the effect of *Malassezia* extracts on IgE production by PBMCs *in vitro*. They found that IgE synthesis by PBMCs from atopic dermatitis patients with specific IgE for *Malassezia* organisms (RAST+) was significantly higher compared with RAST(-) atopic dermatitis patients or normal controls as measured by ELISA. Also, stimulation with *Malassezia* extracts and IL-4

led to a dose-dependent increase in IgE synthesis from PBMCs only in RAST(+) atopic patients, indicating a Th2-type skewed response toward *Malassezia* organisms in these patients (Kröger *et al.* 1995). Several studies comparing the titers of IgE specific to *Malassezia* organisms have revealed similar results. Patients with atopic dermatitis were found to have significantly higher levels of *Malassezia*-specific IgE in their sera compared to those with other atopic diseases or healthy individuals (Young *et al.* 1989; Wessels *et al.* 1991; Nordvall *et al.* 1992; Savolainen *et al.* 2001). Two studies investigating IgE antibodies against *Malassezia* organisms in children and young adults (up to 21 years age) also showed that these antibodies were detected significantly more frequently in those with atopic dermatitis than the other two groups (Nordvall & Johansson 1990; Broberg *et al.* 1992). The *Malassezia*-specific IgE, but not the total IgE in serum, has been found to correlate with the degree of APT response to *Malassezia* extracts at 48 hours post-test in atopic dermatitis patients (Tengvall Linder *et al.* 2000). To the author's knowledge, only one study investigating specific IgE antibodies to *M. pachydermatis* in atopic dogs using an *in vitro* serological test has been documented to date. Significantly higher levels of *Malassezia*-specific IgE, measured by ELISA, were detected in atopic dogs with or without *Malassezia* dermatitis and/or otitis than either healthy dogs or non-atopic dogs with clinical evidence of *Malassezia* overgrowth in the skin and/or ear canals (Nuttall & Halliwell 2001). However, the difference between the atopic groups was not significant (Nuttall & Halliwell 2001). The *Malassezia*-specific IgE antibodies in human and canine atopic patients may play a key role in enhancement of immune responses. The specific IgE antibodies to allergens can bind to Langerhans' cells in the skin, where the allergen capturing and presenting capacity of these APCs can be improved by the membrane-bound IgE upon a second encounter

with the allergen (Mudde *et al.* 1990; Maurer *et al.* 1995).

In contrast to the IgE response to *Malassezia* organisms in atopic human patients, no significant difference in *Malassezia*-specific IgG concentrations was found between adult patients with atopic dermatitis and healthy individuals as measured by *in vitro* serological tests (Tengvall Linder *et al.* 2000; Savolainen *et al.* 2001). However, one study reported significantly elevated *Malassezia*-specific IgG concentrations in young adult patients with atopic dermatitis aged between 16 and 21 years (Broberg *et al.* 1992). The investigators proposed that this probably reflected increased exposure to the organisms through atopic skin and a tendency for IgG to follow IgE production. No correlation has been found between *Malassezia*-specific serum IgG levels and APT responses to the yeast in patients with atopic dermatitis (Tengvall Linder *et al.* 2000). It is, therefore, considered that determination of *Malassezia*-specific IgG concentrations seems to have little value in the diagnosis of *Malassezia* sensitization in atopic human patients (Tengvall Linder *et al.* 2000; Scheynius *et al.* 2002).

It has been demonstrated that atopic dogs with or without cytological evidence of *M. pachydermatis* overgrowth had significantly higher serum titers of *Malassezia*-specific IgG than healthy dogs as measured by ELISA (Nuttall & Halliwell 2001). However, there was no significant difference between atopic dogs with and without *Malassezia* overgrowth (Nuttall & Halliwell 2001). The role of the IgG response to *Malassezia* organisms in the pathogenesis of atopic dermatitis is yet unclear, both in humans and in dogs. IgG antibodies are known to be able to act as opsonins coating microorganisms and to activate phagocytes, which in turn ingest and destroy extracellular pathogens (Janeway *et al.* 1999). This could provide protection for the

host. Nevertheless, IgG antibodies can also activate the complement system, as has been demonstrated with *M. furfur* (Belew *et al.* 1980; Sohnle & Collins-Lech 1983), and produce inflammatory responses (Janeway *et al.* 1999). The precise function of *Malassezia*-specific IgG antibodies remains to be elucidated.

1.4.1.2.4 Mast cell responses

The mast cell response to *Malassezia* antigens has been investigated with intradermal tests (IDT) or SPT in human atopic patients. One study comparing the two test methods with *Malassezia* extracts showed that a higher percentage of patients with atopic dermatitis reacted positively in IDT than in SPT (Young *et al.* 1989). However, positive IDT reactions to *Malassezia* extracts were also seen in some patients with other atopic diseases, whereas all the atopic controls gave negative results in SPT (Young *et al.* 1989). Studies using SPT to detect hypersensitivity to *Malassezia* organisms have shown an increased sensitivity in patients with generalised atopic dermatitis (Young *et al.* 1989; Rokugo *et al.* 1990; Broberg *et al.* 1992) or those with lesions predominantly in the head and neck (Waersted & Hjorth 1985; Kieffer *et al.* 1990; Nissen *et al.* 1998). The SPT results have also been found to correlate with levels of *Malassezia*-specific IgE in the serum (Broberg *et al.* 1992; Kim *et al.* 1999), and with results of basophil histamine release tests (Kieffer *et al.* 1990), but not with the severity of atopic dermatitis (Kim *et al.* 1999).

Positive IDT results to *Malassezia* extracts have also been reported in atopic dogs. Immediate hypersensitivity responses to intradermal injections of *M. pachydermatis* extracts at concentrations which caused no reaction in healthy dogs have been observed in atopic dogs with *Malassezia* dermatitis, although they were also seen in

some atopic dogs without *Malassezia* dermatitis (Morris *et al.* 1998). Nevertheless, the reactivity to the extracts in atopic dogs with cytological evidence of *Malassezia* overgrowth was significantly higher than that in atopic dogs without (Morris *et al.* 1998). Bond *et al.* (2002a) investigated the frequency of IDT reactivity to *M. pachydermatis* extracts in atopic dogs and reported similar results. In this study, atopic dogs were not grouped according to their cytological findings of *Malassezia* populations. However, the frequencies of positive reactivity to the extract in atopic dogs were found to be significantly greater than that in healthy beagles (Bond *et al.* 2002a). In contrast, the frequency of immediate hypersensitivity responses to *M. pachydermatis* extracts in non-atopic dogs with *Malassezia* dermatitis is low. One recent study investigating IDT reactivity to *M. pachydermatis* in 8 healthy basset hounds, 17 basset hounds with *Malassezia* dermatitis, and 19 healthy beagles reported that only two affected basset hounds and one healthy beagle showed immediate hypersensitivity reactions (Bond *et al.* 2002b). Additionally, a preliminary report has demonstrated positive immediate hypersensitivity reactions to extracts from *M. pachydermatis* using passive cutaneous anaphylaxis tests (Morris & DeBoer 2002). Non-atopic dogs received pooled sera from atopic dogs with *Malassezia* dermatitis that were IDT positive to *Malassezia* extracts and serum from an atopic dog with *Malassezia* dermatitis exhibiting high levels of anti-*Malassezia* IgE on an ELISA assay. Positive IDT responses were observed in the recipients following subsequent injection of the yeast extract, indicating that anti-*Malassezia* IgE antibodies are functional in Type I hypersensitivity reactions (Morris & DeBoer 2002). Taken together, these findings suggest that Type I hypersensitivity responses to *M. pachydermatis* allergens may be involved in the pathogenesis and contribute to the clinical signs in some cases of canine atopic dermatitis.



1.4.1.3 Aims

The evidence in the literature suggests that similar pathogenetic mechanisms may occur in canine and human atopic patients with *Malassezia* overgrowth. Although it has been demonstrated that atopic dogs have elevated levels of *Malassezia*-specific IgG and IgE in their serum (Nuttall & Halliwell 2001), the specific IgG or IgE binding proteins from *M. pachydermatis* have not been investigated in these dogs. Hence, the first aim of the studies in this thesis was to characterise the humoral immune responses to *M. pachydermatis* by comparing *Malassezia*-specific IgG and IgE responses to individual antigens of the yeast in atopic dogs, with or without *Malassezia* dermatitis, and normal dogs. The identified allergens could be further purified, analysed, and cloned as recombinant proteins, which could potentially be used for diagnostic purposes and immunotherapy in affected dogs in the future.

1.4.2. Epidermal hyperplasia associated with *Malassezia* dermatitis

The skin acts as a barrier to prevent invasion of microorganisms and its barrier function is largely provided by the epidermis. The process by which the stratum corneum is continually renewed through keratinisation of the epidermal cells may also provide a form of defense against cutaneous microorganisms. It has been considered that epidermal proliferation is important in the defense against superficial mycoses. The renewing process results in continuous shedding of the stratum corneum, which may remove fungal microorganisms (Wagner & Sohnle 1995).

Similar to other chronic inflammatory dermatoses, *Malassezia* dermatitis is often associated with epidermal hyperplasia, which is a protective mechanism seen in the skin in response to a variety of environmental insults. However, the mechanism by which epidermal hyperplasia occurs in *Malassezia* dermatitis is not understood. It could be due to any of the mechanisms described in Figure 1.4. The organism might secrete proteins acting as growth factors and/or interact with surface molecules of keratinocytes, which convey signals to stimulate their proliferation. Alternatively, the organism might play an indirect role and the epidermal hyperplasia could be caused by immune responses stimulated by the yeast or other underlying diseases commonly associated with *Malassezia* dermatitis (section 1.3.3.1). Many of these underlying diseases can cause inflammation and self trauma, which could then lead to the formation of epidermal hyperplasia (Figure 1.4). In the following sections, the process by which basal epidermal cells undergo continued cell division and move the resulting keratinocytes toward the skin surface, and the literature regarding the interaction between keratinocytes and *Malassezia* organisms, are reviewed.

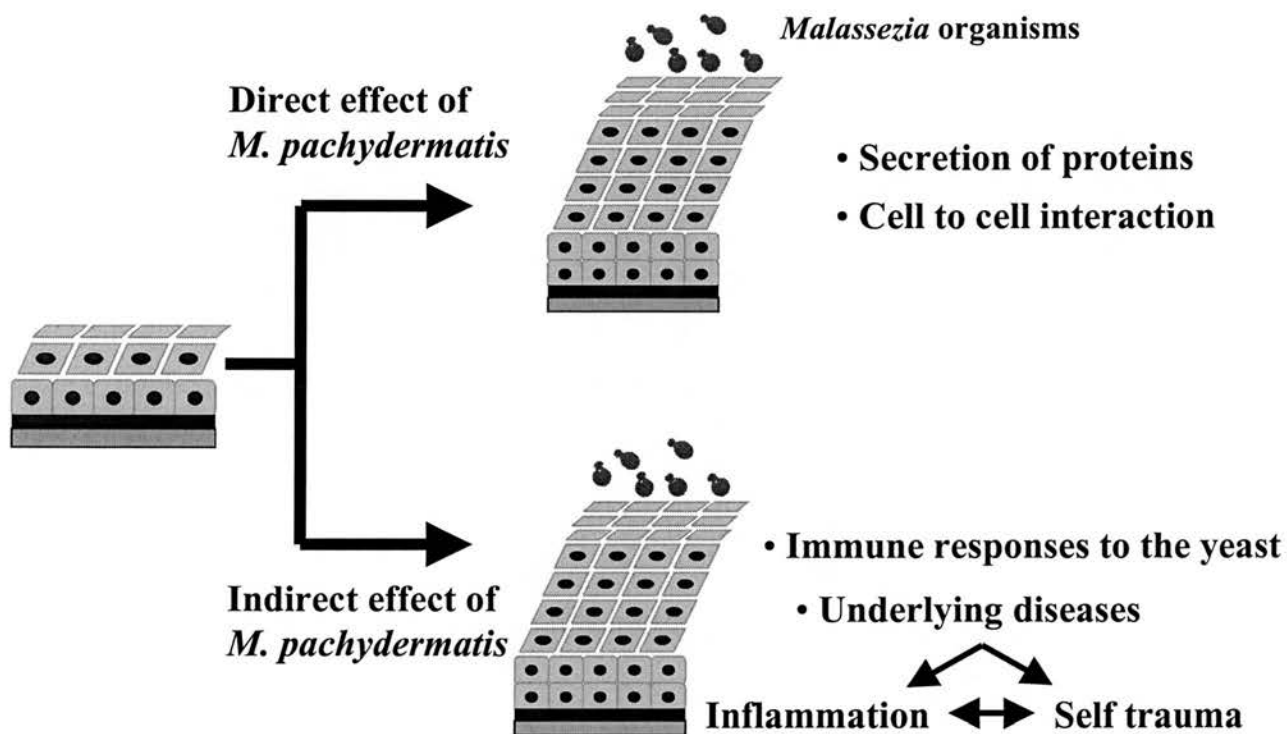


Figure 1.4 Possible mechanisms for the formation of epidermal hyperplasia in *Malassezia* dermatitis

The epidermal hyperplasia seen in *Malassezia* dermatitis might be due to a direct effect of *M. pachydermatis* or an indirect effect of the organism. *Malassezia* organisms might secrete proteins or interact with keratinocytes, which could have a direct effect on their proliferation. Alternatively, *M. pachydermatis* might be an indirect factor in the proliferation of keratinocytes. In this case, the formation of epidermal hyperplasia could be induced by immune responses stimulated by the organism, or inflammation and self trauma caused by other underlying diseases commonly associated with *Malassezia* dermatitis.

1.4.2.1 The process of keratinocyte differentiation

1.4.2.1.1 The structure of the epidermis

The epidermis can be divided into basal (stratum basale), spinous (stratum spinosum), granular (stratum granulosum), and cornified (stratum corneum) layers based on the morphologic features and the state of differentiation the cells assume as they undergo progressive differentiation to form a stratum corneum (Eckert 1989; Credille 2000; Scott *et al.* 2001b). A schematic representation of these layers is shown in Figure 1.5. There are four distinct cell types in the epidermis, including keratinocytes, melanocytes, Langerhans' cells and Merkel's cells, with keratinocytes forming the majority (Scott *et al.* 2001b). The review below focuses on keratinocytes.

Basal layer

The stratum basale is a single row of columnar cells in direct contact with the basement membrane that separates the epidermis from the dermis. Most of these cells are keratinocytes. They contain two different adhesion structures, hemidesmosomes and desmosomes. Hemidesmosomes are located at the junction between basal cells and the basement membrane, and desmosomes mediate adhesion between adjacent basal cells (Eckert 1989). Basal keratinocytes are the only mitotically active keratinocytes in the normal epidermis, and their mitotic activity provides for continual renewal of the epidermis, which is termed proliferation (Kwochka 1993). There is morphologic and functional heterogeneity in basal keratinocytes. Some cells serve primarily to anchor the epidermis to the basement membrane, and others function as stem cells with a proliferative function (Credille 2000; Scott *et al.* 2001b).

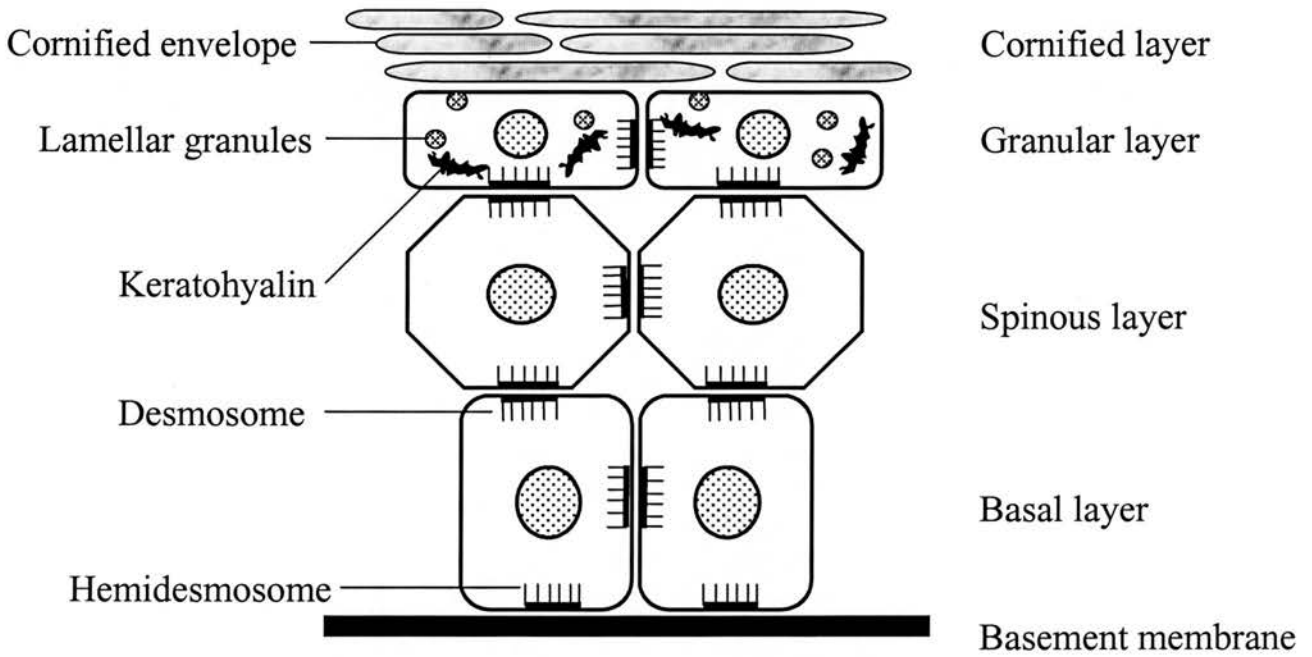


Figure 1.5 Schematic representation of the epidermis

During the process of differentiation, keratinocytes undergo morphological and structural changes. Basal cells are columnar and adhered to the basement membrane. The adhesion complexes at the basal surface of keratinocytes in the basal layer are hemidesmosomes, and those at the junction of adjacent cells are desmosomes. The keratinocytes in the spinous layer are larger and more flattened than basal cells. Granular layer cells contain lamellar and keratohyalin granules. Corneocytes are anuclear and characterised by their cornified envelopes.

Spinous layer

Immediately above the basal layer is the stratum spinosum that is composed of the daughter cells of the basal layer. The keratinocytes in this layer are morphologically larger and more flattened than those in the basal layer (Eckert 1989). Spinous cells contain prominent keratin filament bundles that form a complex intracellular cytoskeleton and function in conjunction with desmosomes to provide stability across the cell layers. The keratinocytes in the spinous layer synthesise lamellar granules (also called membrane-coating granules or Odland bodies) which are filled with lipid (Scott *et al.* 2001b). Lamellar granules are evident in the superficial keratinocytes of the spinous layer although they are more prominent in the cells of the granular layer (Kwochka 1993). They eventually release the lipid contents into the intercellular space, which is important in barrier function of the skin and intercellular cohesion within the stratum corneum (Suter *et al.* 1997).

Granular layer

Above the spinous layer is the granular layer, in which the keratinocytes are thin and flattened with keratohyalin granules in the cytoplasm. Keratohyalin granules are insoluble aggregates of the structural protein profilaggrin, which is synthesised in the granular layer and is the precursor of filaggrin. Filaggrin, an interfilamentous matrix protein, aids in aggregation of keratin filaments into macrofilaments in preparation for the formation of the cornified layer (Suter *et al.* 1997).

Cornified layer

The outermost layer of the epidermis is the stratum corneum. The cornified cells (often called corneocytes) represent the end stage of epidermal differentiation. They

are anuclear, thin, and present in layers (Kwochka 1993). Corneocytes have a highly specialised structure, the cornified envelope, which is formed beneath the plasma membrane during terminal differentiation. The cornified envelope provides structural support to the cell and resists invasion of microorganisms and external injurious agents. Corneocytes are eventually sloughed from the skin surface, and the gradual desquamation is balanced by proliferation of the basal cells, which maintains a constant epidermal thickness (Eckert 1989; Scott *et al.* 2001b).

1.4.2.1.2 Epidermal kinetics

In order to maintain normal epidermal anatomy and physiology, a balance exists between the rate at which new keratinocytes are produced through proliferation in the basal layer and the rate of loss of terminally differentiated cells from the cornified layer. In dogs, the mitotic activity is confined to the basal layer cells that undergo proliferative kinetic cycles, whereas in human skin suprabasilar cells are also mitotically active (Kwochka 1993). A major concept of epidermal kinetics is the “turnover time”, which is the amount of time for the whole cell population to replace itself. This depends both on the time taken for individual cells to divide, the cell cycle time, and the proportion of basal cells dividing, the growth fraction (Eady *et al.* 1998).

The cell cycle is an orderly sequence of events performed by a cell in order to reproduce in which the cell duplicates its contents and then divides into two (Alberts *et al.* 2002a). The cell cycle time represents the interval between two successive mitoses (Eady *et al.* 1998). A proliferating cell goes through a cell cycle which consists of four phases: G₁, S, G₂, and M phases (Figure 1.6). G₁ phase is the post-

mitotic growth phase, in which the cell increases in mass and monitors its environment. S (S for synthesis) phase is one of the two major events of the cell cycle, and it is the phase when DNA duplication occurs. Following S phase is the G2 phase, a pre-mitotic growth phase, in which the cell can further increase its mass before division. M (M for mitosis) phase is the other major phase of the cell cycle in which mitosis occurs and the cell is divided leading to the formation of two daughter cells. The two cells then enter the G1 phase. If extracellular conditions are unfavorable, some cells may delay progress through G1 and enter a resting state known as G0 (Figure 1.6). These cells may be stimulated to re-enter the proliferative cycle by various stimuli. If the extracellular environment is favorable with the presence of stimulating factors, cells in early G1 or G0 progress through a commitment point near the end of G1 known as the restriction point. After passing this point, cells are committed to DNA replication, regardless of whether the stimulating factors are removed. After several proliferative cycles, some cells leave the cycle and undergo terminal differentiation (Eady *et al.* 1998; Hill 2000; Alberts *et al.* 2002a).

The growth fraction is the proportion of basal cells that are proliferative at any one time, which is about 60 % in the normal skin (Eady *et al.* 1998). In dogs, the turnover time for the viable epidermis (stratum basale to stratum granulosum) is approximately 22 days. In wound areas or the skin of dogs with seborrhea, this can be greatly reduced (Scott *et al.* 2001b). High rates of cell production can be achieved by a shorter cell cycle, a higher growth fraction, or both (Eady *et al.* 1998).

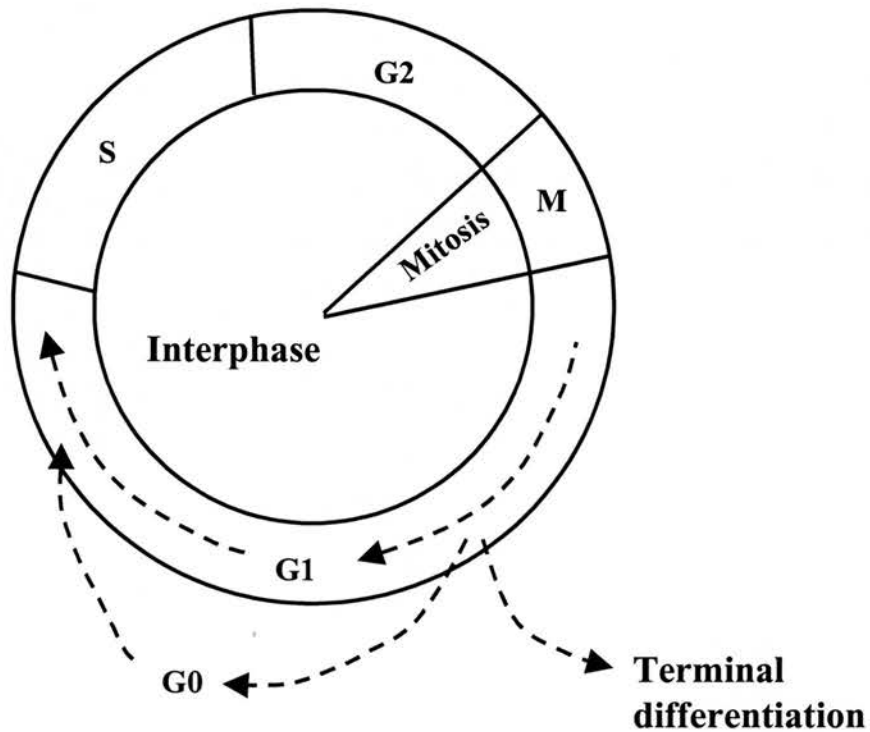


Figure 1.6 The phases of the cell cycle

The cell grows continuously in interphase, which consists of three phases, G1, S, and G2 phases. G1: post-mitotic growth phase; S: DNA synthesis; G2: pre-mitotic phase. In the M phase, the cell undergoes mitosis to generate an identical daughter cell. The cell may also temporarily leave the cell cycle and enter a resting state known as G0 phase, in which it does not proliferate. The cell in G0 phase can re-enter the cell cycle following various stimuli. As some keratinocytes in the basal layer of the epidermis mature, they leave the cell cycle and undergo terminal differentiation.

1.4.2.1.3 The regulatory factors of epidermal proliferation and differentiation

There are a number of factors that have been shown to regulate keratinocyte proliferation and differentiation. These factors are summarised in Table 1.1. The factors that stimulate keratinocyte proliferation will be discussed in further detail in chapter 5 (sections 5.1.1 and 5.6.1).

Table 1.1 Factors known to regulate keratinocyte proliferation and differentiation

Stimulation of proliferation	Inhibition of proliferation/ stimulation of differentiation
<u>Growth factors</u> Epidermal growth factor family Epidermal growth factor (EGF) Transforming growth factor α (TGF- α) Heparin-binding EGF-like growth factor Amphiregulin Epiregulin Betacellulin Neuregulins Fibroblast growth factors (FGF) 1, 2, and 7* Insulin-like growth factor (IGF) 1 <u>Cytokines</u> Interleukin 1 (IL-1) Interleukin 6 (IL-6) Granulocyte macrophage-colony stimulating factor (GM-CSF) Cholera toxin Low concentrations of retinoic acid (10^{-7} – 10^{-6} M)	Transforming growth factor β (TGF- β) Interferon γ (IFN- γ) High calcium concentration <u>Vitamins</u> 1,25-dihydroxyvitamin D ₃ High concentrations of retinoic acid (10^{-6} M) Epidermal chalcones

*FGF 7 is also known as keratinocyte growth factor (KGF)

1.4.2.1.4 Differentiation and desquamation of keratinocytes

Keratinocyte differentiation is the process of re-engineering the basal keratinocyte into a cell capable of forming a rigid structural unit, the corneocyte (Eckert 1989). The signal that causes the initiation of differentiation is currently unclear, but it has been suggested that integrins expressed on basal keratinocytes may play an important role. When occupied by their ligands, integrins hold keratinocytes to the basement membrane. Releasing the ligand conveys a signal to the cell indicating that the attachment to the substratum has been weakened, which could then trigger terminal

differentiation (Blumenberg & Tomic-Canic 1997; Eady *et al.* 1998). During the differentiation process, a basal cell undergoes a series of changes throughout its journey to the skin surface. In addition to the morphological changes described in section 1.4.2.1.1, there are biochemical changes which culminate in the production of corneocytes. Spinous cells produce involucrin, a cornified envelope precursor protein, which is deposited on the inner surface of the plasma membrane of each cell (Rice & Green 1979). They also produce lamellar granules, which will subsequently fuse with the plasma membrane and release lipids into the intercellular spaces of granular and cornified cells (Fuchs 1990). As the spinous cells reach the upper granular layer, they start to produce filaggrin, a protein that allows bundling of keratin filaments into large macrofilaments (Suter *et al.* 1997). Loricrin, a major precursor protein of cornified envelopes, is also synthesised at this stage (Mehrel *et al.* 1990). A calcium influx into the cell activates epidermal transglutaminase, which then catalyses the cross-linking of precursor proteins to form a cornified envelope (Kalinin *et al.* 2001). The end product of this process is the corneocyte, which consists of a cornified envelope enclosing a constellation of keratin macrofibrillar bundles (Eckert 1989). The major events that occur during the final stages of keratinocyte differentiation are summarised in Table 1.2.

Table 1.2 Events during terminal differentiation of keratinocytes

Cleavage of profilaggrin to filaggrin	⇒	Filaggrin binding to keratin filaments	⇒	Filaggrin-dependent assembly of keratin macrofilaments
Activation of proteolytic and nucleolytic activity	⇒	Destruction of cellular organelles, nucleic acids and proteins		
Increased intracellular calcium	⇒	Activation of transglutaminases	⇒	Cross-linking of envelope precursor proteins ⇒ Formation of cornified envelope
Fusion of lamellar granules with plasma membrane	⇒	Release and remodeling of lipid contents	⇒	Formation of extracellular lipid waterproofing layer
Activation of hydrolytic enzymes	⇒	Slough of corneocytes from the skin surface		

Adapted from (Eckert 1989)

1.4.2.2 The role of Malassezia organisms in the pathogenesis of epidermal hyperplasia in man and dogs with diseases associated with Malassezia spp.

There are some similarities between the diseases associated with *Malassezia* organisms in man and dogs. In man, *M. furfur*, *M. globosa*, and *M. sympodialis* have been associated with seborrheic dermatitis, a characteristic clinical feature of which is greasy scales (section 1.3.1.3). Dogs with *Malassezia* dermatitis have greasy skin with varying degrees of scaling (section 1.3.3.3.1) and the histopathology is characterised by marked irregular hyperplasia of the epidermis (section 1.3.3.3.2). Little is known about the direct interaction between *Malassezia* organisms and keratinocytes, either in man or dogs. In a preliminary report, a significant increase in a cellular proliferation marker was demonstrated in canine keratinocytes co-cultured with *M. pachydermatis* compared to control cells *in vitro* (von Tscherner *et al.* 1999). This could indicate that *Malassezia* organisms might be able to activate the proliferative cell cycle of canine keratinocytes. Recently, the epidermal dysplasia in two West Highland White Terriers with cytological evidence of *Malassezia* overgrowth was reported to be reversible after anti-fungal therapy (Nett *et al.* 2001),

suggesting a possible pathogenic role of *M. pachydermatis* in epidermal hyperplasia associated with *Malassezia* dermatitis.

1.4.2.3 Aims

As with other chronic inflammatory dermatoses, skin biopsies from dogs with *Malassezia* dermatitis are characterised by marked epidermal hyperplasia. Elevated populations of *M. pachydermatis* might have a direct effect on the proliferation of canine keratinocytes. The second aim of the studies in this thesis was to investigate the direct effect of *M. pachydermatis* on the proliferation of canine keratinocytes by co-culturing keratinocytes with extracts and culture supernatants from the yeast as well as live *Malassezia* organisms. The results of these studies will provide a better understanding of the pathogenesis of epidermal hyperplasia associated with *Malassezia* dermatitis.

1.5 Summary of aims

The studies described in this thesis were performed with a view to improving our knowledge of the protective mechanisms against *M. pachydermatis* in dogs. They were aimed to investigate two specific aspects of the protective response – activation of a humoral immune response and thickening of the skin by epidermal hyperplasia.

The studies on humoral immune responses were aimed at investigating the antibody responses to *M. pachydermatis* by detecting *Malassezia*-specific IgG and IgE binding proteins from the yeast in atopic dogs with or without *Malassezia* dermatitis and normal dogs. This was achieved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting techniques. The experiments designed to investigate the IgG and IgE responses to *M. pachydermatis* are described in chapters 3 and 4, respectively.

A second aim of these studies was to investigate the role of *M. pachydermatis* in the proliferation of canine keratinocytes. The direct effect of *M. pachydermatis* on keratinocyte proliferation was evaluated in two ways: secretion of proteins by the yeast and interaction between live organisms and keratinocytes. The former was evaluated by culturing keratinocytes with extracts and culture supernatants from the yeast and the latter using an *in vitro* *Malassezia* organism-canine keratinocyte co-culture system. These studies are described in chapters 5 and 6.

Chapter 2

MATERIALS AND METHODS

CLINICAL SAMPLES

2.1 Animals

Clinical samples used in this project comprised serum for immunological studies and skin for keratinocyte culture studies.

Serum samples collected from three groups of dogs were studied. Atopic dogs with *Malassezia* dermatitis and atopic dogs without *Malassezia* dermatitis were recruited from the dermatology clinic at the Royal (Dick) School of Veterinary Studies in Edinburgh. Blood samples were collected with owners' consent and were used for diagnostic purposes in addition to use in this study. Sera of clinically normal dogs were collected from dogs presented for euthanasia at a rescue centre. Most of these dogs were euthanised for behavioural problems as they were deemed unsuitable for re-homing. Eighteen atopic dogs with *Malassezia* dermatitis, 20 atopic dogs without *Malassezia* dermatitis and 18 normal dogs were included in the IgG study. Samples from 28 atopic dogs with *Malassezia* dermatitis and 22 normal dogs were used in the IgE study.

Skin for use in keratinocyte cultures was collected from 17 clinically normal rescue centre dogs immediately post-mortem.

The dogs from which serum and skin were collected comprised a variety of ages, breeds and sexes, but these factors were not studied independently due to the low

number of dogs in each sub-group.

2.1.1 Atopic dogs

The diagnosis of atopic dermatitis was based on a combination of consistent history and clinical signs, exclusion of other causes of pruritic skin disease and the presence of at least one positive intradermal skin test reaction. Coat brushings, skin scrapings and trial therapy were used to rule out ectoparasites. A six-week, home cooked diet trial was conducted to rule out adverse food reactions. No anti-inflammatory medication was given for at least three weeks prior to examination. The intradermal skin tests were performed with 57 allergens (Table 2.1). Following clipping, 0.05 ml of each allergen extract was injected into the lateral flank after sedation with 0.15 mg/kg xylazine intramuscularly. Histamine (1/100,000 w/v) and diluent consisting of phosphate buffered saline with 0.4 % phenol as preservative were included as positive and negative controls, respectively. Test sites were assessed after 20 minutes and scored from 0 to +4 based on erythema, turgidity, elevation of the wheal and wheal size. Scores were assigned by comparing the test sites with the controls. A score of +1 was slightly greater than the negative control, and a +4 approximated the size of the histamine wheal. Reactions ≥ 2 were considered positive (Scott *et al.* 2001c).

2.1.2 Dogs with *Malassezia* dermatitis

Malassezia overgrowth was diagnosed by microscopic observation of Diff-Quik[®] (Dade AG, Switzerland) stained adhesive tape strips (Mason 1992; Bond & Sant 1993; Charach 1997). Samples were obtained from the groin, axilla and interdigital

Table 2.1 Allergens employed in the intradermal skin test

Number	Allergens	Concentration used for skin test	Units	Source
	Dust and Dust mites			
1	<i>Dermatophagoides pteronyssinus</i>	1/1000	w/v	Greer
2	<i>Acaris siro</i>	100	NE/ml	ARTU
3	<i>Dermatophagoides farinae</i>	1/1000	w/v	Greer
4	<i>Tyrophagus putrescentiae</i>	100	NE/ml	ARTU
5	House dust	1000	PNU/ml	Greer
	Epithelial group			
6	Human epithelia	10	µg/ml	ARTU
7	Cat epithelia	100	µg/ml	Greer
8	Horse dander	500	PNU/ml	Greer
9	Sheep epithelia	500	PNU/ml	Greer
10	Mixed feathers	100	µg/ml	ARTU
11	Cotton linters	1/1000	w/v	Greer
	Insects			
12	American/German cockroach	500	PNU/ml	Greer
13	Flea	1000	w/v	Greer
14	Mixed moths	500	PNU/ml	Greer
15	Blackfly (<i>Simulidae</i>)	500	w/v	Greer
16	Mosquito (<i>Culicidae</i>)	500	PNU/ml	Greer
	Trees			
17	White ash	1000	PNU/ml	Greer
18	Ash	1000	NE/ml	ARTU
19	Tag alder	1000	PNU/ml	Greer
20	Alder	1000	NE/ml	ARTU
21	American beech	1000	PNU/ml	Greer
22	Beech	1000	NE/ml	ARTU
23	White oak	1000	PNU/ml	Greer
24	White birch	1000	PNU/ml	Greer
25	Birch	1000	NE/ml	ARTU
26	American elm	1000	PNU/ml	Greer
27	Elm	1000	NE/ml	ARTU
28	Box elder	1000	PNU/ml	Greer
29	Elder	1000	NE/ml	ARTU
30	Plane	1000	NE/ml	ARTU
31	White pine	1000	PNU/ml	Greer
32	Hawthorn	1000	PNU/ml	Greer
	Weeds			
33	Yellow dock	1000	PNU/ml	Greer
34	Jerusalem oak	1000	PNU/ml	Greer
35	Lambs quarter	1000	PNU/ml	Greer
36	Common mugwort	1000	PNU/ml	Greer
37	Nettle	1000	PNU/ml	Greer
38	English plantain	1000	PNU/ml	Greer
39	<i>Brassica spp.</i>	1000	PNU/ml	Greer
40	<i>Chrysanthemum spp.</i>	1000	PNU/ml	Greer
41	Red clover	1000	PNU/ml	Greer
42	Heather	1000	NE/ml	ARTU
	Grasses			
43	Kentucky blue	1000	PNU/ml	Greer
44	Meadow fescue	1000	PNU/ml	Greer
45	Orchard/Cocksfoot	1000	PNU/ml	Greer
46	Perennial rye	1000	PNU/ml	Greer
47	Sweet vernal	1000	PNU/ml	Greer
48	Timothy	1000	PNU/ml	Greer
49	Velvet/Yorkshire fog	1000	PNU/ml	Greer
50	Red top/Bent	1000	PNU/ml	Greer
51	Couch	1000	PNU/ml	Greer
	Moulds/Smuts			
52	<i>Aspergillus mix</i>	1000	PNU/ml	Greer
53	<i>Alternaria tenuis</i>	1000	PNU/ml	Greer
54	<i>Botrytis cinerea</i>	1000	PNU/ml	Greer
55	<i>Penicillium mix</i>	1000	PNU/ml	Greer
56	Barley smut	1000	w/v	Greer
57	Oat smut	1000	PNU/ml	Greer

PNU: protein nitrogen units; NE: nitrogen equivalents.

Greer: Greer Laboratories Inc., Lenoir, NC; ARTU: ARTU Biologicals, Lelystad, The Netherlands.

web and *Malassezia* overgrowth was characterised as an average of 5 or more *Malassezia* organisms per 400X field (Mauldin *et al.* 1997; Scott *et al.* 2001a). Involvement of *Malassezia* in cases of otitis externa was assessed by sampling the external ear canal with a cotton swab and transferring the material to a glass slide prior to heat fixing and staining. The criteria for demonstrating overgrowth were as described for the tape strips.

2.1.3 Normal dogs

Although a full history was not available, the normal dogs had no clinical signs of skin disease and had no lesions on dermatological and otic examination.

2.2 Serum sample collection

Blood samples were collected by cephalic or jugular venipuncture from the three groups of dogs described above and allowed to clot at room temperature. They were centrifuged at 950g for 10 minutes at 4 °C. The harvested sera were stored at -20°C until used.

2.3 Skin sample collection

Skin from clinically normal dogs was collected immediately post-mortem from the dogs presented for euthanasia. The lateral thorax was clipped and prepared aseptically prior to removal of a full thickness skin sample approximately 15 cm x 8 cm (described in detail below in section 2.16).

MALASSEZIA PACHYDERMATIS CULTURE AND PREPARATION

2.4 Culture of *Malassezia pachydermatis*

A single isolate of *M. pachydermatis* was obtained from the ear canal of a dog with *Malassezia* otitis and used throughout these studies. This was deemed appropriate because previous studies have shown that *M. pachydermatis* was the only *Malassezia* species isolated from the skin and external ear canal of dogs with either otitis externa or skin infections, and all isolates of *M. pachydermatis* had similar electrophoretic karyotypes (Senczek *et al.* 1999). Use of a single isolate also allows comparison of multiple serum samples, whereas testing of multiple isolates would require large volumes of serum and reagents. The sample was cultured on Sabouraud Dextrose agar (Oxoid, Basingstoke, UK) containing 20 mg/ml chloramphenicol (Intramycetin[®], Parke-Davis Veterinary, Pontypool, UK) for 48 hours at 37°C, and the colonies were identified as *M. pachydermatis* by microscopic examination. The colonies were then subcultured onto large numbers of plates in order to obtain enough organisms for subsequent studies.

Figure 2.1 shows a plate of *M. pachydermatis* cultured on Sabouraud dextrose agar for 72 hours at 37°C. The yeast colonies appeared smooth, small, white and round. In order to identify the colonies, the following procedures were used to visualise the organisms microscopically. A minute amount of a colony was spread onto a slide with an inoculation loop, fixed over a gentle flame and stained with Gram reagents. Firstly, the slide was stained with crystal violet for 1 minute and then rinsed with water. After staining with iodine for 1 minute followed by another rinse, it was decolourised with acetone and washed with water immediately. The slide was counterstained with 0.1 % basic fuchsin for 30 seconds, rinsed with water and air-



Figure 2.1 Colonies of *Malassezia pachydermatis* on a Sabouraud dextrose agar

A single isolate of *M. pachydermatis* was cultured on Sabouraud Dextrose agar for 72 hours at 37°C. The colonies appeared white, round, and glistening. They had an unpleasant smell when the lid of the petri dish was removed.

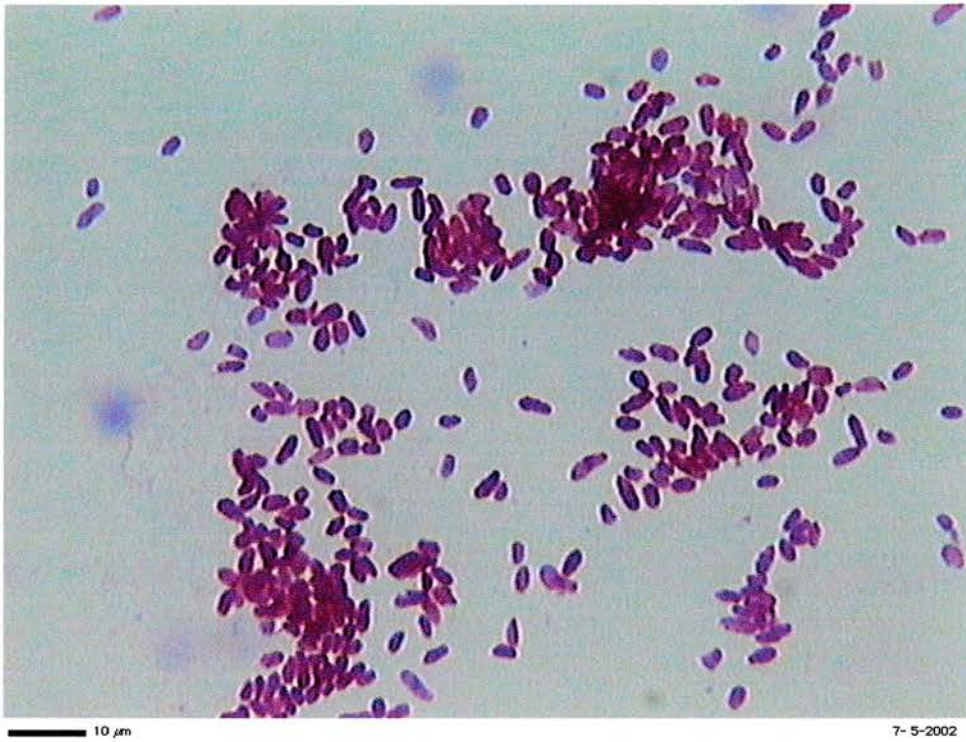


Figure 2.2 Gram stained *Malassezia pachydermatis* organisms (x500)

The yeasts appeared round to oval in shape and about 1-2 x 2-4 μm in size. Budding yeasts are characterised by a peanut shape and a bud collarette.

Scale bar = 10 μm.

dried. The stained *M. pachydermatis* organisms are shown in Figure 2.2.

2.5 Extraction of proteins from *M. pachydermatis*

2.5.1 Reagents and buffers

All the buffers listed below were prepared using de-ionised water. Chemicals were purchased either from Sigma, Poole, UK or BDH, Poole, UK. The phosphate buffered saline (PBS) was autoclaved at 121°C for 1.5 hours if used for tissue culture purposes. *Malassezia* extraction buffer was prepared either with or without protease inhibitors.

PBS	<i>Malassezia</i> extraction buffer
8.5g NaCl (Sigma)	988.25 mg NH ₄ HCO ₃ (Sigma)
2.7g Na ₂ HPO ₄ ·12H ₂ O (BDH)	Protease inhibitors:
0.4g NaH ₂ PO ₄ ·2 H ₂ O (BDH)	262.4 mg ε-aminocaproic acid (Sigma)
	186 mg ethylenediaminetetraacetic acid (Sigma)
	17.4 mg phenylmethylsulfonyl fluoride (BDH)
Dissolved in 1 liter water	Dissolved in 100 ml water
pH 7.2-7.3	pH 7.4

2.5.2 Optimisation of the extraction procedure

Malassezia colonies were carefully harvested and suspended in PBS for a washing procedure that consisted of three cycles of centrifugation at 500 g for 5 minutes followed by removal of the supernatant and resuspension in PBS. After the last washing cycle, the cells were resuspended in extraction buffer (pH 7.4) comprising 125 mM NH₄HCO₃ and protease inhibitors (20 mM ε-aminocaproic acid, 5 mM ethylenediaminetetraacetic acid and 1 mM phenylmethylsulfonyl fluoride) (Jensen-

Jarolim *et al.* 1992; Morris *et al.* 1998).

Preliminary experiments were performed using three different methods to determine the optimum method for protein extraction. First, the *Malassezia* colonies in the extraction buffer were mixed vigorously with an equal volume of glass beads (0.4 mm, 40 mesh, BDH, UK) on a vortex mixer for 10 minutes to mechanically disrupt the cell membranes. A second suspension of *Malassezia* organisms was subjected to three cycles of freezing and thawing by alternately immersing the container in a dry ice-methanol mixture and warm tap water. This was intended to fracture the cell membranes and allow release of cytoplasmic contents. Thirdly, a *Malassezia* suspension was placed in an ultrasonic water bath for 5 minutes in order to physically fragment the organisms. Ten agar plates were prepared for each method, and equal weights of colonies (3.07 gm) were added to 5 ml of extraction buffer. After extraction, the cell suspensions were stored at 4°C overnight to allow equilibration of cellular proteins. The suspensions were then centrifuged at 6000 g for 5 minutes and the supernatants were collected. The amount of protein obtained from each method was measured with BCA Protein Assay Reagent (see section 2.8), and the extracts were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (see section 2.10). The protein concentrations of the extracts obtained from the three methods (glass beads, freeze/thaw cycles, and ultrasonification) were 16.35 mg/ml, 0.58 mg/ml, and 0.22 mg/ml, respectively. Figure 2.3 shows the Coomassie blue stained protein profiles of the three different extracts on an SDS-PAGE gel (see section 2.11 for Coomassie blue staining). Much stronger protein bands were seen in the extract obtained by glass bead disruption. This method was therefore used in subsequent studies.

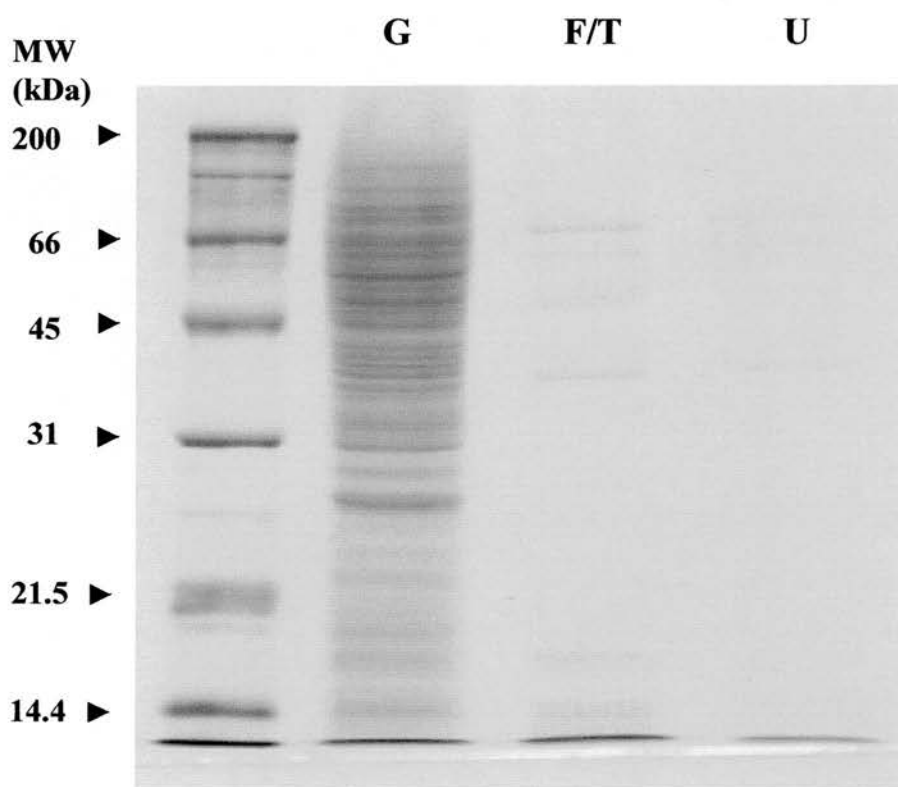


Figure 2.3 Extracts of *M. pachydermatis* obtained using three different methods in a Coomassie blue-stained SDS-PAGE gel

G: Glass beads; F/T: Three freeze/thaw cycles; U: Ultrasonification. Molecular weights in kilodaltons. The extract produced using glass bead disruption was selected for the subsequent studies.

The effect of glass bead disruption was further assessed by examining Leishman's stained cytopsin preparations of the remaining cell pellets. The pellets were resuspended in PBS and 100 μ l of the suspension was centrifuged for 5 minutes at 600 rpm onto a glass slide in a cytocentrifuge (Shandon Cytospin 2, Southern Instruments LTD., Runcorn, UK). The slide was air-dried and then stained with Leishman's solution (Fisher Scientific, Loughborough, UK) for 2 minutes. An equal volume of distilled water was added to the slide and it was stained with diluted Leishman's solution for a further 8 minutes. After rinsing in tap water, the slide was air-dried and mounted with DPX mountant (Fisher Scientific, UK). Figure 2.4 shows the *Malassezia* pellets stained with Leishman's solution. Substantial amounts of cell debris were present in the pellets. The majority of the organisms were destroyed by glass beads and showed defective features.

For some experiments, the extract was prepared using buffer without protease inhibitors. The *Malassezia* extracts prepared with or without protease inhibitors using glass bead disruption were compared in an SDS-PAGE gel (see section 2.10 for SDS-PAGE) and shown in Figure 2.5. The two extracts exhibited very similar protein profiles with some variations of density shown by individual bands.

2.6 Culture supernatants from *M. pachydermatis*

Culture supernatants from *M. pachydermatis* were prepared by inoculating the organisms into Sabouraud liquid medium (Mast Laboratories Ltd, Merseyside, UK) and then culturing them for three days at 37°C. The cell suspension was then centrifuged at 6000 g for 5 minutes and the supernatant collected. It was concentrated using Centriplus™ concentrators (Millipore Corporation, Bedford, MA,

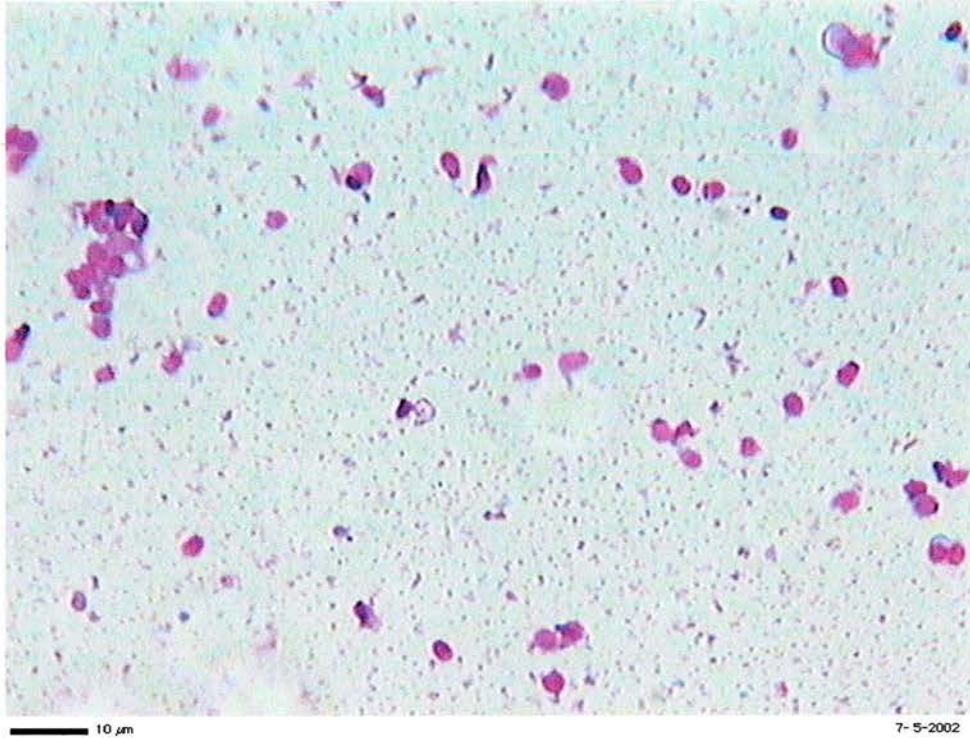


Figure 2.4 Leishman's stained *Malassezia pachydermatis* cell pellets obtained after glass bead disruption (x500)

Considerable amounts of cell debris resulted from glass bead disruption. Very few *Malassezia* organisms retained their intact shape with the majority exhibiting abnormal morphology.

Scale bar = 10 μm.

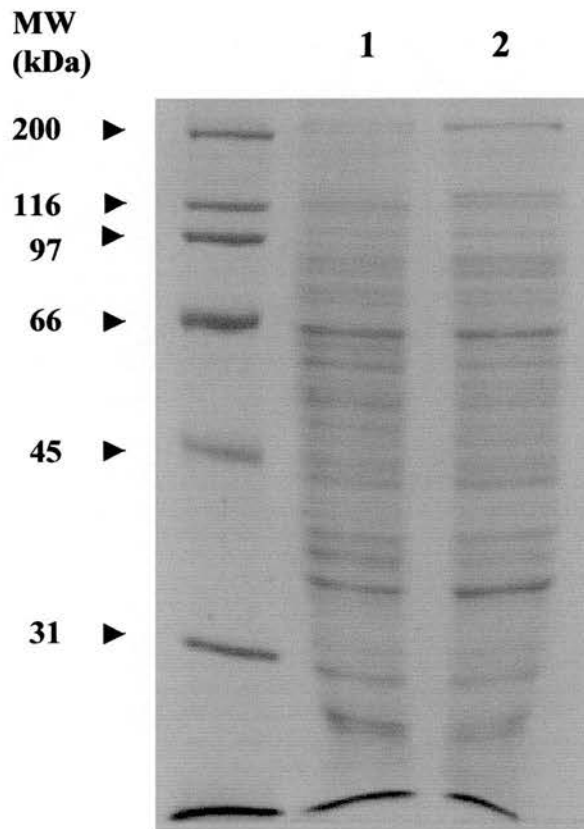


Figure 2.5 Coomassie brilliant blue stained extracts of *Malassezia pachydermatis* on an SDS-PAGE gel

Lane 1: *Malassezia* extracts produced with protease inhibitors.

Lane 2: *Malassezia* extracts produced without protease inhibitors.

The molecular weight markers on the left are 200, 116, 97, 66, 45, and 31 kDa.

USA) to a final volume of 2 ml by repeated centrifugation at 3000g until the required volume was reached. An equal volume of sterile medium was also incubated and concentrated in parallel to act as a control. The protein profile of the culture supernatants was checked using an SDS-PAGE gel (section 2.10) with reducing sample buffer after sterilisation (see section 2.7) and is shown in Figure 2.6. No visible bands were present in the sterile medium. On the contrary, culture supernatants from *Malassezia* showed faint visible bands between 66 and 45 kDa, indicating that proteins within this molecular weight range were generated in the liquid medium or released from *Malassezia* during culture.

2.7 Sterilisation of *Malassezia* extracts and supernatants

For experiments requiring sterile materials, extracts and culture supernatants from *M. pachydermatis* were sterilised using syringe filters (Nalgene®, Nalge Nunc International Corp., New York, USA). The *Malassezia* extracts were centrifuged at 6000 g for 5 minutes and then at 20,000 g for 10 minutes to further purify the extracts and facilitate filtration. They were then sterilised by passing through syringe filters with a 0.8, 0.45 and 0.2 µm pore size serially. A 50 µl aliquot of the filtered extracts was plated on Sabouraud Dextrose agar and the plate was incubated for 3 days at 37°C to check its sterility. No growth of any organisms was observed after the three-day incubation.

The culture supernatants from *Malassezia* were sterilised by passing through syringe filters with a 0.2 µm pore size. The sterility of the cell supernatant and control media was checked by incubating 50 µl of each on Sabouraud Dextrose agar as described above.

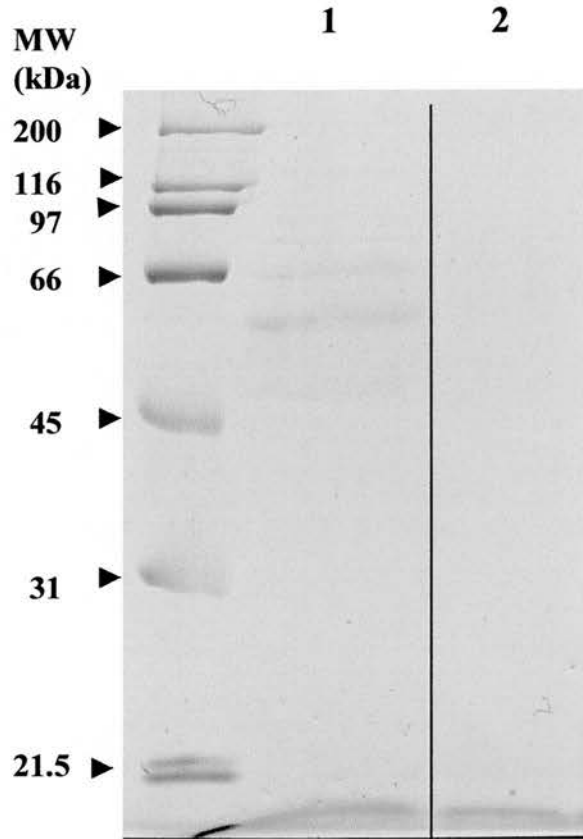


Figure 2.6 Coomassie brilliant blue stained culture supernatants from *M. pachydermatis* on an SDS-PAGE gel

Lane 1: culture supernatants from *Malassezia*. Faint bands are visible.

Lane 2: sterile Sabouraud liquid medium. No bands are visible.

The molecular weight markers on the left are 200, 116, 97, 66, 45, 31, and 21.5 kDa.

2.8 BCA protein concentration assay

The assay reagent was obtained from Pierce Chemical Company, Rockford, IL, USA. Protein standards were prepared by two-fold dilution of the 2.0 mg/ml BSA stock standard with PBS. 10 µl of each standard and sample was added to the wells of a 96 well plate in triplicate followed by 100 µl of working reagent. After incubating for 30 minutes at 37°C, the plate was read at 570 nm in a Dynatech ELISA plate reader (Dynatech MR5000, Dynatech Laboratories Ltd, Billingshurst, UK). The protein concentrations of samples were determined using a linear standard curve generated by analysing the O.D. values of protein standards.

GEL ELECTROPHORESIS AND IMMUNOBLOTTING

2.9 Reagents and solutions

The following solutions were prepared with de-ionised water unless stated otherwise. Chemicals were obtained from various companies including Bio-Rad, Hemel Hempstead, UK; Fisher Scientific, UK; National Diagnostics, Hessele Hull, UK; and Sigma, UK.

Resolving buffer (1 M Tris/HCl)	Stacking buffer (0.5 M Tris/HCl)
12.1g Tris (Sigma)	6.05g Tris
In 100 ml water	In 100 ml water
Adjusted to pH 8.8 with HCl	Adjusted to pH 6.8 with HCl

Sodium dodecyl sulphate (SDS, 10 %)	Ammonium persulphate (10 %)
10g SDS (Fisher Scientific)	100 mg Ammonium persulphate (Bio-Rad)
In 100 ml water	In 1 ml water

	Separating gel		Stacking gel
	10 %	12 %	4 %
Resolving buffer	2.5 ml	2.5 ml	
Stacking buffer			1.4 ml
Acrylamide (National Diagnostics)	3.35 ml	4.1 ml	0.72 ml
Water	3.05 ml	2.3 ml	2.95 ml
10 % SDS	1 ml	1 ml	0.28 ml
TEMED (Sigma)	10 μ l	10 μ l	10 μ l
10 % Ammonium persulphate	100 μ l	100 μ l	100 μ l

Sample buffers	Reducing	Non-reducing
Stacking buffer (see above)	1.25 ml	1.25 ml
Glycerol (BDH)	1 ml	1 ml
10 % SDS	2 ml	2 ml
β - mercaptoethanol (Sigma)	0.5 ml	
Distilled water	5 ml	5.5 ml
0.05 % (w/v) Bromophenol blue (Sigma)	0.25 ml	0.25 ml

Running buffer (10X)	Tris buffered saline (TBS)
72.05g Glycine (Fisher Scientific)	2.42g Tris
15.14g Tris	29.24g NaCl
5g SDS	
In 500 ml water	In 1 liter water
pH 8.3	pH 7.5

CAPS transfer buffer (10 mM CAPS in 10 % methanol)	Towbin transfer buffer
1.1065g 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS, Sigma)	1.515g Tris
50 ml methanol (Fisher Scientific)	7.2g Glycine
	100 ml methanol
In 500 ml water	In 500 ml water

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

The broad range molecular weight standards (Bio-Rad, UK) were diluted 1: 20 in reducing sample buffer and 10 μ l aliquots were stored at -20°C until used. The separating polyacrylamide gel and stacking gels were prepared as described in section 2.9. The minigel apparatus (Bio-Rad, UK) was set up as described in the manufacturer's instructions and the gels were poured between the glass plates. A minigel was made of 3.5 ml of separating gel solution overlaid by 1.5-2 ml of stacking gel solution that covered the bottom of the teeth of a comb used to generate the wells. Two different combs were used in this study. A comb with multiple teeth was used for experiments that required loading several different samples in the same gel. For immunoblotting that required strips containing equal amounts of *Malassezia* extracts, a comb with only two teeth was used to generate a regular-sized well for molecular weight standard and a wide well for *Malassezia* extracts. The gel was allowed to set for 30 minutes and the comb was removed. Samples were diluted 1:1 with either reducing or non-reducing sample buffer and then heated at 95°C for 5 minutes along with an aliquot of thawed molecular weight standard. They were then added into wells on the top of the gel and the gel electrophoresis was performed in the running buffer at 200 V for either 40 minutes (10 % separating gel) or 60 minutes (12 % separating gel).

2.11 Coomassie blue staining

Both staining and destaining were performed on a platform rocker. For gels which were not used for immunoblotting, the separated proteins and molecular weight standards were visualised by staining gels with Coomassie blue solution comprising

0.25 % Coomassie brilliant blue R (BDH, UK), 10 % acetic acid (Fisher Scientific, UK) and 45 % methanol (Fisher Scientific, UK) for 5-10 minutes. The gels were then destained with 10 % acetic acid and 45 % methanol to remove background. With several changes of destain solution, it usually took 2-3 hours to obtain clear bands with a clean background.

2.12 Electrophoretic transfer

After the electrophoresis was completed, the separated proteins and molecular weight standards were transferred from the gel to a polyvinylidene difluoride microporous membrane (Millipore ImmobilonTM-P Transfer Membrane, Millipore Corporation, Bedford, MA) in a Bio-Rad Trans-Blot[®] SD Semi-Dry Electrophoretic Transfer Cell. A piece of membrane about the same size as the gel was immersed in 100% methanol for 3 seconds and then equilibrated in the transfer buffer. CAPS transfer buffer (LeGendre *et al.* 1993) was used for 12 % separating gels and Towbin transfer buffer for 10 % separating gels. Four pieces of Whatman chromatography paper cut to the same size as the gel were pre-wetted in the transfer buffer. The gel and membrane were then sandwiched between them as illustrated in Figure 2.7. The transfer was run at 80 mA per minigel for 1 hour. The quality of transfer was checked by staining gels and molecular weight standards blotted onto the membrane with Coomassie brilliant blue R-250 for 5-10 minutes followed by destaining with several changes of destain solution as described in section 2.11.

2.13 Immunoblotting

Precise details of the reagents, conditions and dilutions used for immunoblotting can be found in Chapters 3 and 4. Briefly, the membrane (whole or cut into appropriately

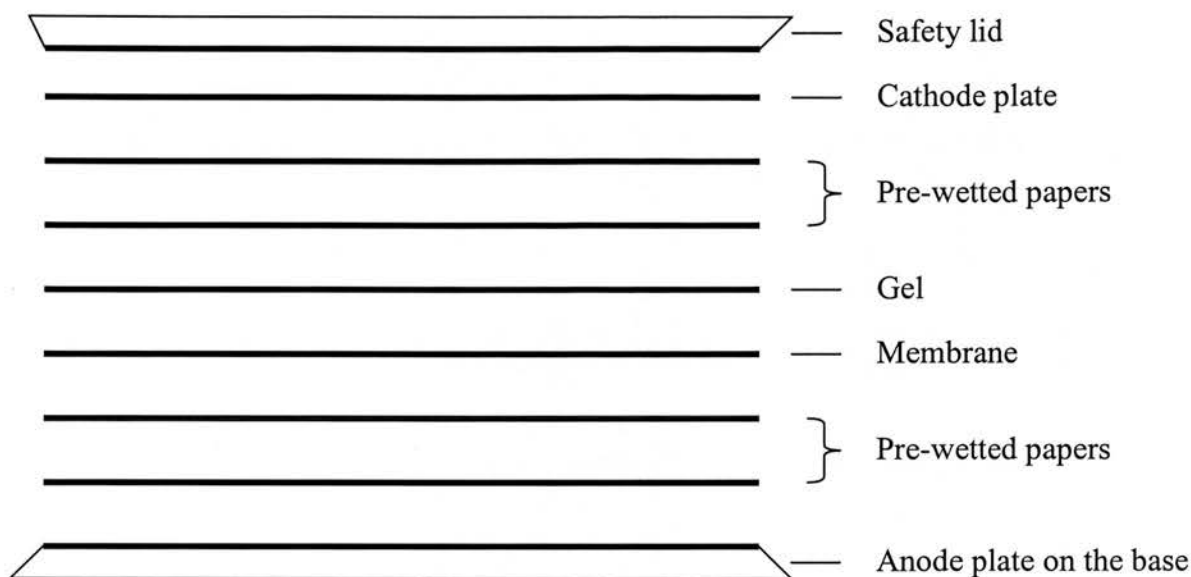


Figure 2.7 Profile of the gel sandwich in a Bio-Rad Trans-Blot® SD semi-dry electrophoretic transfer cell

Two sheets of pre-soaked paper were placed onto the platinum anode and a tube was rolled over the surface of the paper to exclude air bubbles. The rolling step was repeated every time a layer was added to ensure there were no bubbles between each layer. The pre-wetted membrane was then placed on top of the paper followed by the gel on the top of the membrane. Another two sheets of pre-soaked paper were placed on top of the gel and the cathode was carefully placed onto the stack. The safety cover was placed on the unit before connecting it to the power supply.

0.5 cm x 7 cm strips) was pre-wetted in 100% methanol for 3 seconds and rinsed with water for a few minutes. It was then blocked for 1 hour with blocking reagents including various concentrations of skimmed milk and goat sera, casein solution (Vector Laboratories Inc., Burlingame, CA, USA), Tween 20 (Fisher Scientific, UK), Tween 80 (Sigma, UK), and an avidin/biotin blocking kit (Vector Laboratories Inc., Burlingame, CA, USA). After washing with TBS containing 0.05 or 0.1 % Tween 20 (Fisher Scientific, UK) (TTBS) for 15 minutes (3 × 5 minute rinses), the membrane was incubated with 1/5 – 1/100 diluted dog serum samples in dilution buffer (1 % skimmed milk in TTBS) for 30-60 minutes. It was then washed again in TTBS for a further 15 minutes. Bound IgG or IgE was detected by incubating the membrane for 1 hour with enzyme conjugated anti-dog IgG or IgE antibodies diluted in dilution buffer followed by a 15-minute wash with TTBS. If the anti-dog IgG or IgE antibody was not conjugated, a further 1-hour incubation with an enzyme conjugated secondary antibody was used followed by washing with TTBS for 15 minutes. The membrane was developed with appropriate substrates either chromogenically or chemiluminescently. The molecular weights of bands detected were determined using standard curves generated by analysing the position of molecular weight markers on Coomassie blue stained membranes (section 2.11).

CULTURE OF CANINE KERATINOCYTES AND FIBROBLASTS

2.14 Reagents and solutions

The following solutions were obtained from Gibco™ Invitrogen life technologies, Paisley, UK: Penicillin (10,000 units/ml) and Streptomycin (10,000 µg/ml); Fungizone (250 µg/ml); Dulbecco's Modified Eagle medium with GlutaMAX™ I; William's Medium E; dispase; 10 x Trypsin/EDTA; Murine epidermal growth factor.

Foetal calf serum was batch tested and purchased from Gibco™ Invitrogen life technologies or Sigma, UK. Trypan blue (0.4%), cholera toxin and hydrocortisone were obtained from Sigma.

2.15 Cell culture plastics and incubation conditions

Unless stated otherwise, cells were cultured in plastic tissue culture treated flasks or various multi-well plates (see specific sections), all of which were obtained from Corning Costar, Bucks, UK. All cultures were maintained at 37°C in a humidified atmosphere with 5 % CO₂ in air.

2.16 Primary keratinocyte culture

Healthy skin was taken immediately post-mortem from dogs presented for euthanasia. The flank of dogs was shaved and scrubbed with Hibiscrub (ZENECA Limited, Macclesfield, UK), painted with Povidone-Iodine and wiped with 70% ethanol. Full-thickness skin (approximately 15 cm x 8 cm x 0.5 cm) was removed and dipped in Povidone-Iodine/ PBS solution (1:5) for 10 seconds and then washed with PBS containing Penicillin (100 units/ml), Streptomycin (100 µg/ml), and Fungizone (2.5 µg/ml) (PSF). The skin was then immersed in Dulbecco's Modified Eagle Medium (DMEM) containing PSF.

Canine keratinocyte culture was established using a modification of a protocol published previously (Wilkinson *et al.* 1987). The subcutaneous tissue and fat were removed by trimming and the skin was cut into small pieces (approximately 1 cm x 1 cm). The pieces were then incubated in William's Medium E containing 10 mg/ml dispase and PSF overnight at 4°C. The epidermis was carefully stripped off the

dermis and placed in a solution of Trypsin/EDTA (0.1 %/0.04 %) at 37°C for 10 minutes. The enzymatic effect of trypsin was stopped by adding an excess of William's Medium E supplemented with 10% foetal calf serum (FCS), 0.1 nM cholera toxin, 10 ng/ml murine epidermal growth factor, and PSF (complete medium). The cell suspension was filtered with a cell strainer (70 µm, Becton Dickinson, Oxford, UK) to remove hair and cell debris. It was then centrifuged at 700g for 10 minutes and the cells were resuspended in complete medium. For primary cultures, tissue culture flasks coated with fibronectin (kindly provided by Dr. R. C. McKenzie, University of Edinburgh, UK) at a concentration of 1 µg/cm² were used to enhance cell attachment to the plastic. The cells were counted in a haemocytometer and the viability was assessed by trypan blue exclusion (section 2.14). Cells were then plated into 25 cm² tissue culture flasks at a density of 1-3 x 10⁶ viable cells per flask.

2.17 Cell counting and viability

Cells were counted in an improved Neubauer haemocytometer after mixing 10 µl of cell suspension with an equal volume of trypan blue. This counting procedure is based on the principle that viable cells exclude the stain and dead cells take it up. Viability was calculated by dividing the number of viable cells into the total cell number.

2.18 Primary fibroblast culture

Skin pieces were rinsed with PBS containing PSF after the epidermis was stripped off. They were then immersed in Trypsin/EDTA (0.1 %/0.04 %) at 37°C for 45 minutes and the container was shaken occasionally during the incubation. Excess

DMEM supplemented with 10% FCS and hydrocortisone (0.4 µg/ml) was added to terminate the enzyme reaction and the cells were collected by filtering the supernatants through a cell strainer. The suspension was centrifuged at 700g for 10 minutes and the cells were resuspended in supplemented DMEM. They were counted as described above and plated at 1.2×10^5 viable cells/cm².

2.19 Subculture

In order to obtain more cells for subsequent experiments and avoid early differentiation, cells were subcultured when they reached approximately 80 % confluence. The cell monolayer was washed with sterile PBS (warmed to 37°C) after the media was removed. An appropriate volume of Trypsin/EDTA diluted in PBS (0.1 %/0.04 %) was added and the cells were incubated at 37°C until they separated from the plate. By gently tapping the edges of a flask or plate using the palm of a hand, it usually took 15-30 minutes for the keratinocytes and less than 10 minutes for the fibroblasts to separate. The completion of trypsinisation was determined by observation of a single cell suspension (not in strings or clumps) microscopically. Media containing 10 % FCS was added 1: 1 into the cell suspension and the cells were spun down by centrifugation at 700g for 10 minutes, resuspended in fresh media and counted. The suspension was adjusted to a desired cell concentration before placing into a new container.

CHARACTERISATION OF KERATINOCYTE CULTURES

2.20 Cultivation of cells on cover slips and chamber slides

Keratinocytes and fibroblasts were subcultured onto cover slips or chamber slides for immunostaining studies to visualise their growth and cellular characteristics. Circular

glass cover slips with a diameter of 22 mm were obtained from Fisher Life Science, Loughborough, UK and 8 well Nunc Lab-Tek™ II chamber slides from Merck Eurolab Ltd, Lutterworth, UK. The latter are tissue culture treated glass slides with a removable medium chamber. After culturing was complete, the medium chamber was removed to allow the slides to be processed as standard microscope slides.

Cover slips were sterilised by dipping in 70 % alcohol and air-dried in the tissue culture hood. They were carefully inserted into wells of a 12 well plate. Each cover slip was coated with 10 µg of fibronectin (kindly provided by Dr. R. C. McKenzie, University of Edinburgh, UK) in PBS and left for 30 minutes at room temperature. Excess solution was aspirated and the cover slips were air dried before use. Skin cells were plated at a density of 5×10^5 viable cells/well, and confluence occurred in 4-5 days. No coating was applied to chamber slides, and cells were cultured at 2×10^4 viable cells/cm² (1.4×10^4 cells/well) for immunostaining studies (see section 2.28).

2.21 Fixation

Media was removed from wells and they were washed with PBS three times to remove non-adherent cells. Cells were then fixed in 4 % paraformaldehyde in PBS for 20 minutes at room temperature. Excess paraformaldehyde was aspirated and the cells were washed with PBS three times. Cover slips and chamber slides were preserved in 70 % alcohol to prevent putrefactive changes and stored at 4°C until used.

2.22 Preparation of solutions for staining

The following solutions were used for haematoxylin & eosin staining (section 2.23)

and immunohistochemistry studies (section 2.24, 27- 28). They were prepared using chemicals obtained from either Fisher Scientific or Sigma.

Citrate buffer (10 mM)	Tris buffered saline (TBS)	Scott's tap water substitute
2.1g citric acid·H ₂ O	6.06g Tris	3.5g NaHCO ₃
	8.77g NaCl	20g MgSO ₄
In 1 liter distilled water	In 1 liter de-ionised water	In 1 liter tap water
Adjusted to pH 6.0 with NaOH	Adjusted to pH 7.6	Checked pH 8

2.23 Haematoxylin & Eosin (H & E) staining

H & E staining was carried out on cells cultured on cover slips and preserved in 70 % alcohol. The cover slip was washed with tap water and soaked in Harris haematoxylin (Surgipath, Peterborough, UK) for 1 minute followed by Scott's tap water substitute for 30–60 seconds. It was then washed with tap water three times and stained with eosin for 30 seconds. After washing again in tap water three times, the cover slip was dehydrated using absolute ethanol with five changes and then xylene with two changes. It was then carefully removed from the well and mounted upside down with DPX mountant (Fisher Scientific, UK).

Figure 2.8 shows the result of keratinocytes stained with H & E. Cells were present with purple-staining nuclei and pink-staining cytoplasm. They tended to vary in size depending on the degree of proliferation. Newly reproduced cells were smaller compared to other cells. Occasional cells showed mitotic features and one or two cells appeared to be apoptotic or necrotic.

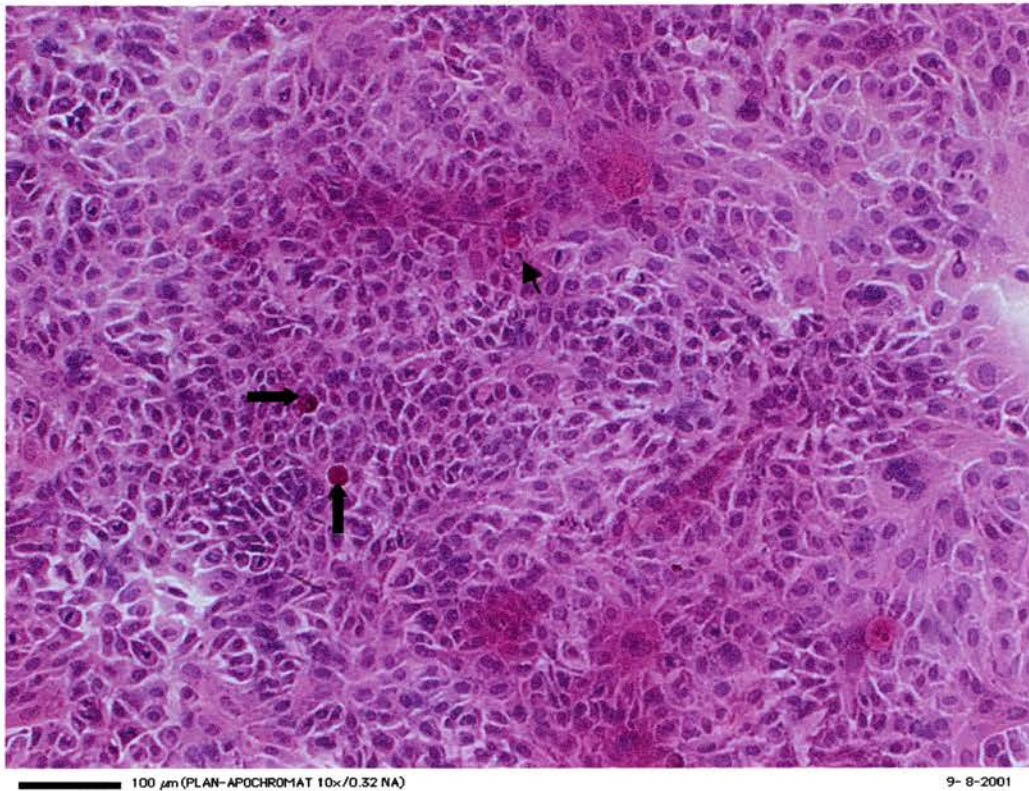


Figure 2.8 H&E stained canine keratinocytes (x100)

Canine keratinocytes were subcultured onto a cover slip coated with fibronectin and cultured to confluence. Some cells were still undergoing mitosis (thin arrow). Apoptotic or necrotic-like cells showed dark red staining cytoplasm and pyknotic nuclei (bold arrows). Newly reproduced cells appeared to be smaller than other cells (areas around arrows).

Scale bar = 100 μm .

2.24 Cytoskeleton detection

Canine keratinocyte and fibroblasts were mixed 1: 1 and cultured at 2×10^4 viable cells/cm² in chamber slides for three days before fixation. To inhibit intrinsic peroxidase activity, the cells were treated with 1 % H₂O₂ in methanol for 20 minutes. They were then rinsed with PBS and incubated with 1% SDS in PBS for 5 minutes. After washing with PBS, the slides were assembled with coverplates and placed in a slide rack (SequenzaTM, Thermo Shandon Inc, Pittsburgh, USA). The reagents of the Vector Elite ABC mouse IgG kit (Vector Laboratories Ltd., Peterborough, UK) were added according to the manufacturer's instructions and the immunostaining was performed at room temperature. Cells were blocked with horse serum diluted in PBS for 1 hour and then incubated with monoclonal mouse antibody against broad spectrum keratin, or vimentin (EURO/ DPC Ltd., Gwynedd, UK) for a further one hour. Cells incubated with universal monoclonal negative control antibody (EURO/ DPC Ltd., Gwynedd, UK) were used as a control. They were washed again in PBS and then incubated with biotinylated horse anti-mouse IgG for 30 minutes. ABC reagents were added after another wash with PBS and allowed to react for 30 minutes. The slides were rinsed with PBS and developed using DAB (3, 3'-diaminobenzidine, Vector Laboratories Ltd., Peterborough, UK) for 7 minutes. The development was ceased by washing the slides with distilled water and they were then removed from the slide rack and counterstained with Mayer's haematoxylin (Sigma, UK) for 5 minutes followed by two rinses in Scott's tap water substitute. The slides were dehydrated by dipping serially in 70 %, 90 %, 95 % and 100 % ethanol for 5 minutes each and then clarified in xylene for another 5 minutes. Excess xylene was wiped off from the back of the slides and they were mounted with DPX mountant.

Figure 2.9 shows the cells stained with anti-keratin, anti-vimentin, and control Abs. Keratinocytes reacted to anti-keratin Ab and exhibited brown staining, but fibroblasts appeared blue, the colour of the counterstain (Figure 2.9 A). In contrast, the cytoplasm of fibroblasts stained positively with anti-vimentin Ab unlike keratinocytes which were negative (Figure 2.9 B). All the cells stained blue if the primary Ab was replaced with the negative control Ab (Figure 2.9 C) indicating that the brown staining present was specific.

EVALUATION OF CELL GROWTH

2.25 Keratinocyte proliferation assay

A colourmetric assay reagent (Celltiter 96® AQueous One Solution Cell Proliferation Assay from Promega, UK) was used to determine the number of viable keratinocytes in the study assessing the effects of extracts and culture supernatants of *M. pachydermatis* on canine keratinocyte proliferation. The validation of this assay is described in Chapter 5. The assay was performed in 96 well tissue culture plates. Keratinocyte monolayers were trypsinised, washed and counted in a haemocytometer. Cells were diluted with media and plated both in the 96 well plates (100 µl/well, 2×10^4 viable cells/cm²) and flasks, in which cells were grown to generate a standard curve for the cell proliferation assay. Various treatments were applied to the cells in the plates during culture (see chapter 5). When performing the assay, cells grown in the flasks were trypsinised, washed and counted in a haemocytometer. A volume of 100 µl of cell suspension (containing two fold dilutions from 2×10^5 to 3125 cells per well) was added in triplicate to allow generation of a standard curve. Wells with 100 µl of medium only were included as background. After addition of 20 µl of Celltiter 96® AQueous One Solution reagent to the wells containing growing

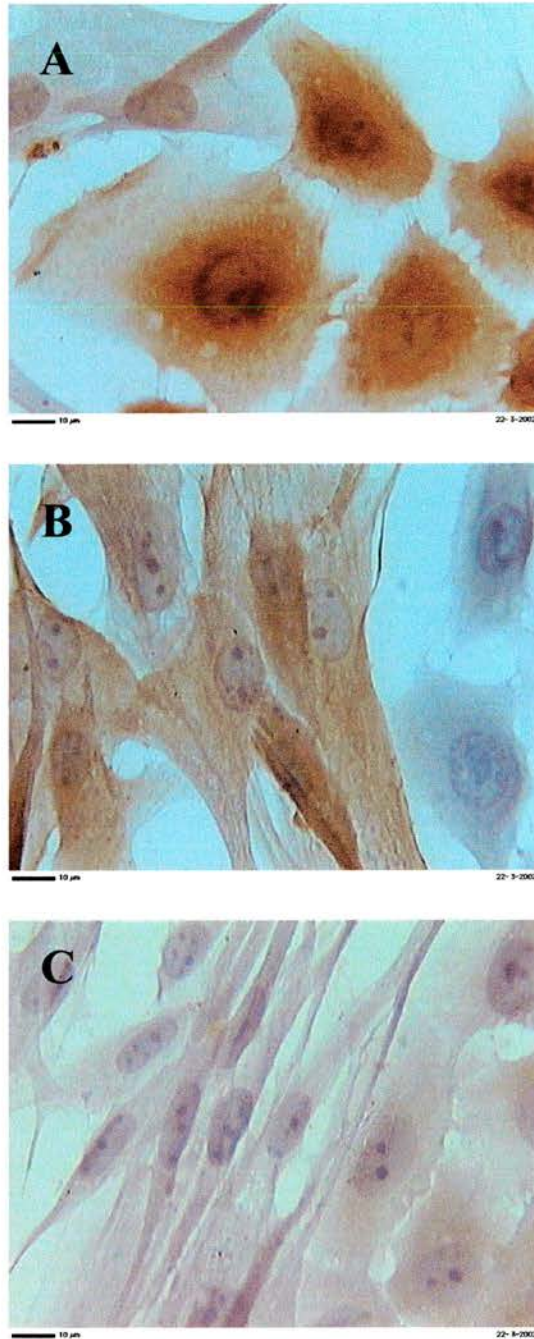


Figure 2.9 Cytoskeleton staining in canine keratinocytes and fibroblasts (x500)

A: Anti-keratin Ab stained keratinocytes showing dark brown staining compared to adjacent fibroblasts.

B: Silk- like vimentin in the cytoplasm of fibroblasts stained with anti-vimentin Ab. Keratinocytes appeared unstained.

C: Both keratinocytes and fibroblasts were negatively stained using control Ab.

Scale bars = 10 µm

keratinocytes, background and standards, the plates were incubated at 37°C for a further 3 hours. The absorbance of the reaction was evaluated using an ELISA reader (Dynatech MR5000, Dynatech Laboratories Ltd, Billingshurst, UK) at a wavelength of 490 nm. A reference wavelength of 630 nm was applied simultaneously to reduce background generated by excess cell debris, fingerprints and other nonspecific absorbance.

2.26 Statistical analysis

The specific tests employed will be described in the appropriate chapters.

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2.27 Antigen retrieval

Keratinocytes cultured on chamber slides for proliferating cell nuclear antigen (PCNA) and Ki-67 antigen detection were subjected to antigen retrieval treatments to improve the immunoreactivity of antigens and to enhance staining. For PCNA detection, 10 mM Citric buffer (pH 6.0) was boiled by microwaving in a pressure cooker and slides rinsed with TBS were put in a slide rack and placed in the citric buffer (see section 2.22 for buffer preparation). The pressure cooker was heated with the lid on at high power until the pressure valve rose and then it was heated for a further 5 minutes at pressure.

For Ki-67 antigen detection, slides were rinsed in distilled water with two changes and immersed in 10 mM citric buffer in a glass beaker that was heated on a hot plate until it boiled. The buffer was kept boiling for 10 minutes and the beaker was removed from the hot plate and allowed to cool down for at least 10-20 minutes at

room temperature.

2.28 Staining procedures

2.28.1 Proliferating cell nuclear antigen detection

After antigen retrieval treatment (section 2.27), the slides were incubated with 1 % H_2O_2 in distilled water for 5 minutes. They were then rinsed with distilled water and placed in TBS for a further 5 minutes. The immunostaining was performed in a slide rack and the slides were incubated with horseradish peroxidase conjugated anti-PCNA antibody (Dako, Cambridgeshire, UK) for 1 hour at room temperature. Negative control slides were processed by replacing the anti-PCNA Ab with a horseradish peroxidase conjugated immunoglobulin negative control (Dako, Cambridgeshire, UK). They were then rinsed with TBS and developed with DAB for 5 minutes. The counterstain, dehydration and mount were carried out in the manner described in section 2.24.

2.28.2 Ki-67 antigen detection

The antigen retrieval procedures were first applied to the slides (section 2.27) and they were then rinsed with PBS and incubated with 1 % H_2O_2 in distilled water for 5 minutes. After rinsing with distilled water, the slides were placed in PBS for 5 minutes. The immunostaining was performed in the same way as for the cytoskeleton in a slide rack. Anti-Ki-67 monoclonal Ab (Zymed, San Francisco, USA) diluted 1:50 with PBS was used as the primary Ab and a negative control slide was incubated with mouse primary Ab isotype control (Zymed).

2.28.3 Apoptosis detection

The slides were immersed in PBS for 5 minutes and then placed in a slide rack for immunostaining. The apoptotic cells were detected using DermaTacs™, an *in situ* apoptosis detection kit for skin cells and tissues (R & D Systems Europe, Oxon, UK). The staining was performed at room temperature unless stated specifically and the manufacturer's instructions were followed. The slides were incubated with Cytonin as a permeabilization and blocking agent for 30 minutes and then rinsed with distilled water. After incubating with 3 % H₂O₂ in methanol for 5 minutes, they were washed with PBS and incubated with terminal deoxynucleotidyl transferase enzyme (TdT) labelling buffer for 5 minutes. The labelling reaction mix containing brominated deoxynucleoside triphosphate (B-dNTP) mix, TdT enzyme and TdT labelling buffer was then added and allowed to react for 45 minutes at 37°C. A positive control was generated by adding nuclease into the labelling reaction mix and a negative control was generated by omitting the TdT enzyme. The labelling reaction was stopped by adding TdT stop buffer and the slides were washed with PBS after 5 minutes. They were then incubated with biotinylated mouse monoclonal anti-bromodeoxyuridine (BrdU) antibody for 45 minutes at 37°C and then washed with PBS. Bound BrdU was detected by incubating the slides with HRP conjugated streptavidin for 10 minutes and washed with PBS followed by distilled water. The colour was developed with TACS blue Label™ for 5 minutes and the slides were rinsed with distilled water before counterstaining in eosin-based Red Counterstain C for 5 minutes. After further washing in distilled water, the slides were air-dried, clarified by dipping in xylene for 5 minutes and mounted with DPX mountant.

2.29 Cell counting

All the slides were counted blind. 100 cells were counted in randomly chosen confluent areas at high magnification (400X) and the nuclei were regarded as PCNA or Ki-67 positive if the nuclei showed any specific brown staining. The whole area of a well on the chamber slide was scanned for apoptotic cells at 100 X magnification and cells that were condensed and exhibited increased Red Counterstain C uptake or showing blue nuclear staining were regarded as apoptotic.

Chapter 3

CHARACTERISATION OF THE IgG RESPONSE TO *MALASSEZIA PACHYDERMATIS* ANTIGENS IN ATOPIC AND NORMAL DOGS

3.1 Introduction

It has been well characterised that atopic diseases are clinical manifestations of Type I hypersensitivity reactions mediated by IgE antibodies (Platts-Mills 2001). IgG antibodies may also be involved in the disease process, however, their role in atopic disease is less well defined in contrast to IgE. Both a pathogenic and a protective role have been suggested for IgG antibodies in Type I hypersensitivity. This introduction will focus on the relationship between IgG antibodies and atopic diseases in human beings and dogs.

3.1.1 Anaphylactic IgG antibodies

The first indications of the existence of a heat-stable anaphylactic IgG antibody were demonstrated approximately 30 years ago using serum samples from human patients with food allergy (Parish 1970). These heat-stable antibodies were shown to be able to sensitise skin mast cells of monkeys and were not removed from the serum by anti-IgE immunosorbent. Furthermore, they could be precipitated by anti-IgG. Unlike the long lasting sensitisation seen with IgE, which peaks at 24 hours, the sensitisation with heat-stable IgG reached maximum in approximately 2 hours and persisted for less than 24 hours (Parish 1970). They were therefore called “short-term sensitising IgG”, IgG-(S-TS). However, due to the lack of reliable *in vitro* assays to detect IgG-(S-TS), its precise identity has not been determined (Van der Zee &

Aalberse 1991).

In dogs, anaphylactic IgG antibodies (IgGd) with similar properties to IgG-(S-TS) in man were first reported by Willemse *et al.* (1985a). Allergen specific IgGd were detected most frequently against house dust, human dander, grass pollens and spring tree pollens in atopic dogs using an ELISA technique (Willemse *et al.* 1985b). In a later study, an IgGd response to a 90 kDa polypeptide in crude extracts from *Dermatophagoides farinae* was demonstrated in atopic dogs by Western blotting (Noli *et al.* 1996). Nevertheless, high levels of IgGd to allergens of *Dermatophagoides farinae* and *D. pteronyssinus* were also detected in normal dogs (Lian & Halliwell 1998). It may be concluded from these data that the role of IgGd in canine atopic disease is still uncertain.

3.1.2 IgG response to environmental allergens

In addition to IgE responses, the correlation between IgE and IgG responses to specific environmental allergens, as well as allergen recognition by IgG subclasses, have been studied in man. Using purified allergens of house dust mite and rye grass pollen in an antigen-binding assay, it has been shown that there is a strong correlation between IgE and IgG responses to Der p I and Rye I (Chapman & Platts-Mills 1978; Platts-Mills *et al.* 1978). Barnes *et al.* (1993) showed that IgG responses to antigens of house dust mites were predominately associated with IgG1 antibodies in children with atopic dermatitis as demonstrated by ELISA. However, a similarity of antibody binding epitopes in grass pollen extracts (*Dactylis glomerata*) was found to lie between IgE and IgG4 by Desvaux *et al.* (1989) using immunoblotting techniques to characterise specific IgE, IgA, IgM classes and IgG subclasses in patients allergic to

grass pollen. Furthermore, significantly higher concentrations of total and allergen-specific IgG4 in sera were observed in patients with atopic dermatitis as compared to normal individuals (Shakib *et al.* 1977; Merrett *et al.* 1984) whereas the levels of specific IgG1 antibodies to house dust mite and grass pollen in atopic patients were not significantly different from those of non-atopic individuals (Kemeny *et al.* 1989). Although there are similarities in biological activity between IgG-(S-TS) and IgG4, there has been no clear evidence to conclude that IgG4 has anaphylactic activity and is IgG-(S-TS) (Parish 1981).

Both total and allergen-specific IgG responses to environmental allergens have also been documented in dogs. Significantly higher total IgG concentrations were observed in atopic dogs than in normal dogs, as measured by radial immunodiffusion assays (Hill *et al.* 1995). Atopic dogs were also shown to have higher concentrations of allergen-specific IgG than non-atopic dogs as detected by ELISA (Hites *et al.* 1989). Day *et al.* (1996) showed that allergen-specific IgG antibodies were most frequently detected to *D. farinae*, *D. pteronyssinus*, mould mix, insect mix, and meadow fescue among 33 allergens included in a commercial ELISA test kit. In the same study, the IgG subclass profile of allergen-specific antibodies was found to be allergen-dependent with the IgG response to *D. farinae* and *D. pteronyssinus* being dominated by IgG4 antibodies, and Timothy grass predominantly within the IgG1 and IgG4 subclasses (Day *et al.* 1996). The literature regarding the humoral immune response in dogs with flea allergy dermatitis is inconsistent. One study showed that dogs with flea bite hypersensitivity had higher levels of both IgE and IgG antibodies to partially purified flea antigen than flea naive dogs and non-atopic dogs exposed chronically to fleas, as measured by radioimmunoassay (Halliwell & Longino 1985).

In contrast, McKeon and Opdebeeck (1994) showed that neither IgG nor IgE reactivity detected using ELISA or western blotting could distinguish between dogs with and without flea allergy.

3.1.3 IgG response to allergen-specific immunotherapy

IgG antibodies are generally considered important in the treatment of atopic diseases in regard to allergen-specific immunotherapy. Elevated IgG responses have been observed in human patients undergoing allergen-specific immunotherapy with IgG1 antibody appearing first and shifting to an IgG4-dominated responses later (Djurup & Osterballe 1984; McHugh *et al.* 1990; Peng *et al.* 1992). However, both positive and negative correlations between IgG4 responses and clinical improvement during immunotherapy have been documented (Nakagawa *et al.* 1983; Djurup & Osterballe 1984; Hedlin *et al.* 1986; Malling & Djurup 1988). The protective role of IgG4 as a blocking antibody that competes for antigen with anaphylactic antibody in Type I hypersensitivity still remains uncertain.

Although substantially less research on the correlation between IgG responses and immunotherapy has been done in dogs compared to human beings, similar findings have been reported. One study showed that atopic dogs not only had higher concentrations of allergen-specific IgG than non-atopic dogs, it also increased further after specific allergen immunotherapy (Hites *et al.* 1989).

3.1.4 Antigen-specific IgG response to cutaneous microorganisms

Cutaneous microorganisms such as *Staphylococcus* spp. bacteria and *Malassezia* yeasts are known to be important in the pathogenesis of atopic dermatitis, both in

man and in dogs. Higher levels of colonisation of *Staphylococcus aureus* and *Pityrosporum (Malassezia)* species are commonly seen in human patients with atopic eczema (Ring *et al.* 1992). Likewise, dogs with atopic dermatitis frequently exhibit concurrent skin infection with *Staphylococcus intermedius* or *Malassezia* yeasts (DeBoer & Marsella 2001).

The IgG responses to these microorganisms have been studied less extensively in man than IgE responses. One recent study reported that atopic children under 7 years of age more frequently had IgG antibodies to *Staphylococcus aureus*-derived superantigen, enterotoxin B, than normal controls. Also, the levels of these specific IgG antibodies measured by ELISA were higher, although they did not correlate with serum IgE concentrations (Campbell & Kemp 1998). In contrast, no difference in the levels of *Malassezia*-specific IgG was shown between patients with atopic dermatitis and healthy controls (Tengvall Linder *et al.* 2000; Savolainen *et al.* 2001). These two studies were carried out in patients with a median age of over 25 years. Interestingly, a study looking at IgG antibody responses to *Malassezia* organisms on skin of children and young adults with atopic dermatitis aged between 0-21 years showed that young adults (16-21 years) had significantly elevated *Malassezia*-specific IgG levels (Broberg *et al.* 1992).

It has been shown that atopic dogs with *Staphylococcus intermedius* skin infection (pyoderma) had significantly higher anti-staphylococcal IgG concentrations in their sera compared to normal dogs, using an ELISA to measure antigen-specific IgG (Morales *et al.* 1994; Shearer & Day 1997a). Nine major IgG binding epitopes of *Staphylococcus intermedius* ranging between 25 and 120 kDa were identified by

Western blotting analysis in groups of dog with various skin diseases including atopic dermatitis with and without pyoderma (Shearer & Day 1997a). Elevated serum titers of *M. pachydermatis*-specific IgG measured by ELISA have also been found in dogs with skin diseases associated with *M. pachydermatis* (Bond *et al.* 1998) and atopic dogs with *Malassezia* overgrowth (Nuttall & Halliwell 2001). In addition, four proteins of 219, 110, 71 and 42 kDa in extracts of *M. pachydermatis* have been demonstrated using Western blotting to be recognised mainly by non-atopic dogs with *Malassezia* dermatitis as compared to healthy dogs (Bond & Lloyd 2002). However, specific IgG binding proteins in *M. pachydermatis* have not been investigated using sera of atopic dogs.

3.1.5 Aims

The aim of this part of the study was to characterise one arm of the humoral immune response to *M. pachydermatis* by comparing the IgG binding activities to individual antigens of the yeast in atopic dogs with and without *Malassezia* dermatitis, and normal dogs in order to further clarify the role the organism plays in atopic dogs. The initial objective of the experiments in this chapter was to develop a Western blotting technique to detect specific IgG binding antigens of *M. pachydermatis*. This method was to be used to analyse all subsequent serum samples. The first part of this chapter describes the experiments performed to validate and optimise the Western blotting technique. The second section describes the detection of IgG binding antigens of *M. pachydermatis* in three groups of dogs using the optimal conditions demonstrated in the former section.

VALIDATION AND OPTIMISATION OF METHODOLOGY

3.2 Specificity of anti-canine IgG antibody

Aims

In order to characterise the IgG response to *Malassezia pachydermatis*, it was essential to demonstrate that the anti-dog IgG used in this study bound to IgG in dog sera specifically. The aim of the initial experiments in this section was to validate the feasibility of using a horseradish peroxidase conjugated goat anti-dog IgG heavy and light chain (Bethyl Laboratories, Montgomery Tx, USA) for immunoblotting.

3.2.1 Binding of anti-canine IgG antibody to purified canine IgG

Methods

Purified dog IgG (Sigma, UK), a normal dog serum sample, and mouse IgG1 κ (Sigma, UK) were separated in a 12 % SDS-PAGE gel and blotted onto a membrane which was then incubated with the goat anti-dog IgG to test its specificity. The samples were diluted 1/100, 1/100, and 1/10 respectively with PBS and mixed 1:1 with reducing sample buffer before being subjected to electrophoresis. A volume of 20 μ l from each sample was loaded into a minigel along with 5 μ l of molecular weight standards, and the SDS-PAGE was performed as described in chapter 2 (sections 2.9 and 2.10). After electrophoresis, one gel was stained with Coomassie blue (section 2.11) and the other was used for electrophoretic transfer and immunoblotting (sections 2.12 and 2.13).

The molecular weight standards transferred onto the membrane were cut off and stained with Coomassie blue. The rest of the membrane was blocked for 1 hour with 1 % skimmed milk in TBS. After washing with TBS containing 0.05 % Tween 20

(TTBS) for 15 minutes (3 x 5 minute rinses), the membrane was incubated for 1 hour with a horseradish peroxidase conjugated goat anti-dog IgG heavy and light chain (Bethyl Laboratories, Montgomery Tx, USA) diluted 1/5,000 in dilution buffer (1 % skimmed milk in TTBS). The membrane was washed again in TTBS for a further 15 minutes and then developed with DAB (3, 3'-diaminobenzidine) peroxidase substrate (Vector Laboratories, Inc., Burlingame, CA, USA) for about 5 minutes. After rinsing with water to remove excess substrate, the membrane was air-dried.

Results

The protein profiles of purified dog IgG, normal dog serum and mouse IgG1 κ in an SDS-PAGE gel are shown in Figure 3.1 A. As the electrophoresis was performed under reducing condition, purified dog IgG was cleaved into heavy and light chains which have a molecular weight of approximately 54 and 28 kDa, respectively. These two bands were also present in the normal dog serum and mouse IgG1 κ .

The binding of the anti-dog IgG to heavy and light chains of canine IgG is shown in Figure 3.1 B. Two bands were seen in the lanes of purified dog IgG and normal dog serum, which corresponded to the two bands at the same molecular weight range shown in the gel. However, no bands were in the lane of Mouse IgG or to any of the other proteins present in the dog serum sample indicating that the anti-dog IgG was specific for canine IgG.

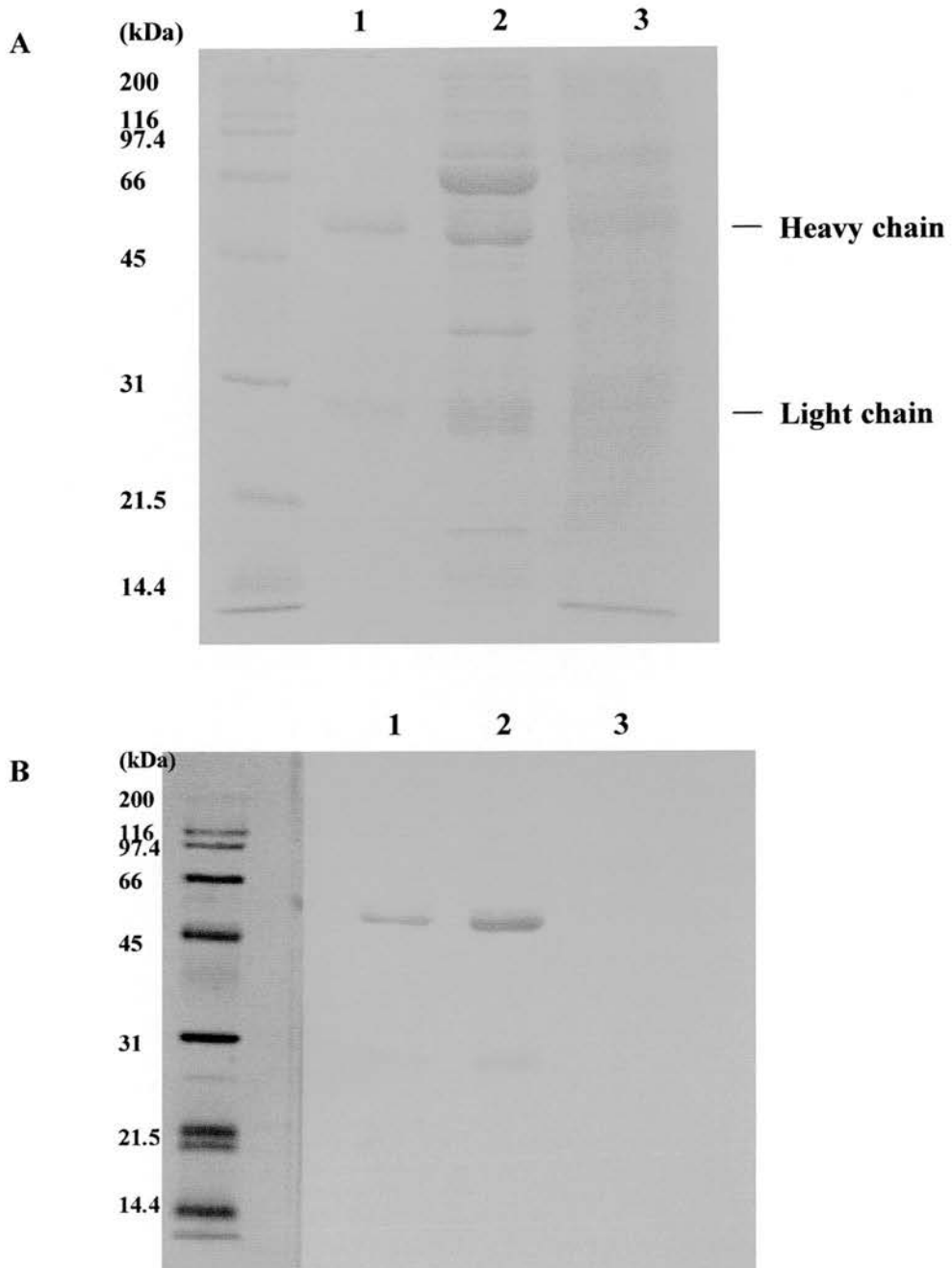


Figure 3.1 A-B Demonstration of specific binding between the horseradish peroxidase conjugated goat anti-dog IgG and purified canine IgG

A: Purified canine IgG (lane 1), a serum sample from a normal dog (lane 2) and mouse IgG1 κ (lane 3) analysed using reducing sample buffer in an SDS-PAGE gel. Purified canine IgG was cleaved into two distinct bands: a heavy and light chain. Multiple protein bands were present along with the heavy and light chains of IgG in dog serum. More than two bands were found in mouse IgG1 κ . These may include the heavy chain of mouse IgG1, the light chain of IgG1 and their breakdown products.

B: Specificity of the horseradish peroxidase conjugated goat anti-dog IgG demonstrated by immunoblotting. Two bands were detected both in purified canine IgG and dog serum, whereas no visible bands were detected in mouse IgG1 κ , confirming that the anti-dog IgG was specific for heavy and light chains of canine IgG.

3.2.2 Specificity of the anti-canine IgG antibody in *Malassezia* immunoblots

The previous experiment demonstrated that the anti-dog IgG was specific for canine IgG. However, it was not known at this stage whether the Ab would bind to IgG in dog sera specifically and not cause non-specific binding to *Malassezia* extracts. Since the conclusions of this part of study were to be drawn from the binding shown on a membrane, it was important to determine that the Ab bound to antigenic components in *Malassezia* extracts rather than non-specific binding between the anti-dog IgG and the extracts.

Methods

The *Malassezia* extract prepared with glass beads (123 µg, 150 µl of 1/20 dilution) was mixed with reducing sample buffer and a final volume of 300 µl was added into a broad well running across the top of the gel. The electrophoresis was run at 200 V for 60 minutes and the proteins were transferred to a membrane as described in chapter 2. This allowed the membrane to be cut into narrow strips so that they could be probed with individual sera or different reagents. The strips were dipped in methanol for a few seconds and rinsed in de-ionised water. It was found that this step helped achieve an evenly spread colour development.

An 8-channel incubation tray was used to hold the strips and reagents. Each channel held a strip and 1 ml of reagents. Eight strips were divided into two groups: the strips in group one (no. 1-4) were blocked with 1 % skimmed milk in TBS, and strips in group two (no. 5-8) were incubated with TBS (Figure 3.2). The first two strips in each group were then incubated with different dilutions of dog sera (1/25 or 1/100), whereas the other two were used as controls and incubated only with dilution buffer.

Figure 3.2 Experimental design

Strips	1	2	3	4	5	6	7	8
Blocking	1 % skimmed milk in TBS				None			
Serum	1/25	1/100	Buffer	Buffer	1/25	1/100	Buffer	Buffer
Anti-IgG	1/1,000							

The strips were incubated with either 1 % skimmed milk in TBS or plain TBS for 1 hour followed by a 15 minute wash (3 x 5 minute rinses) in TBS containing 0.05 % Tween 20 (TTBS). A serum sample from an atopic dog with *Malassezia* dermatitis was diluted either 1/25 or 1/100 in dilution buffer (1 % skimmed milk in TTBS) and allowed to react with the strips for 30 minutes. After another 15-minute wash, bound IgG was detected by incubating the strips for 1 hour with anti-dog IgG diluted 1/1,000 in dilution buffer. The strips were washed again in TTBS and developed with DAB peroxidase substrate.

Results

The strips incubated with dog sera showed intense staining (Figure 3.3), but the bands were difficult to interpret due to the dark background. This could stem from insufficient blocking, high concentrations of serum or anti-dog IgG. Further optimisation of the reagents was therefore required in order to allow clear visualisation of the bands. However, despite the high background, no staining developed in strips incubated only with dilution buffer, regardless of whether or not they were blocked. This indicated that the anti-dog IgG did not bind to *Malassezia* extracts non-specifically, thus confirming that the brown staining obtained was generated from IgG in dog sera which had bound to *Malassezia* extracts.

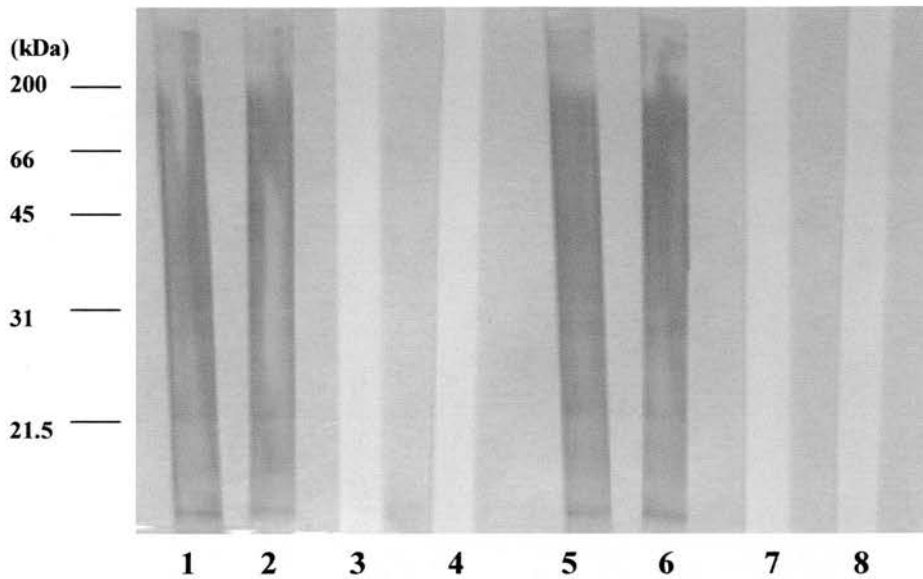


Figure 3.3 Specificity of the anti-canine IgG in *Malassezia* immunoblots

Immunoblots showing the effects of blotting and different serum dilutions. Lanes 1-4 were blocked with 1 % skimmed milk in TBS whereas lanes 5-8 were not blocked. Lanes 1 and 5 were incubated with dog serum diluted 1/25, and lanes 2 and 6, 1/100. The strips incubated with serum (lanes 1, 2, 5 and 6) showed intense staining, although distinct bands were not visible. In contrast, the strips incubated with buffer alone showed no binding (lanes 3, 4, 7 and 8), indicating that the anti-dog IgG did not bind to *Malassezia* extracts non-specifically.

3.3 Optimisation of blocking reagents

Aims

Due to the difficulties in visualising bands on the strips in the previous experiment, different concentrations of blocking reagents were used to reduce the background.

Methods

As the anti-dog IgG was purified from goat serum, two different dilutions of normal goat serum were used as a blocker in addition to skimmed milk. Eight strips with separated *Malassezia* proteins were pre-wetted with methanol, rinsed with water and blocked with either skimmed milk or goat sera diluted 1/100 or 1/20 in TBS for 1 hour (Figure 3.4). They were washed with TTBS for 15 minutes (3 x 5 minute rinses) and then incubated with dog sera. Serum samples were used from an atopic dog with *Malassezia* dermatitis and a normal dog and included in each blocker group tested. Due to the intense binding seen in the previous experiment, the dog sera and anti-dog IgG were used at higher dilutions in this experiment. The strips were incubated with dog sera diluted 1/100 in dilution buffer for 30 minutes followed by washing in TTBS for 15 minutes. They were then incubated for 1 hour with anti-dog IgG diluted 1/5,000, washed again in TTBS for 15 minutes and developed with DAB peroxidase substrate.

Figure 3.4 Experimental design

Strips	1	2	3	4	5	6	7	8
Blocking	1 % milk		5 % milk		1 % goat serum		5 % goat serum	
Serum	AM 1/100	N 1/100	AM 1/100	N 1/100	AM 1/100	N 1/100	AM 1/100	N 1/100
Anti-IgG	1/5,000							

AM: atopic dog with *Malassezia* dermatitis; N: normal dog.

Results

The comparison between different blocking reagents is shown in Figure 3.5. There was substantially less staining shown in strips incubated with the normal dog serum compared with the atopic dog serum, suggesting that the brown staining shown in the atopic dog was a specific antibody-antigen reaction rather than non-specific background. Despite this, no difference was observed between each blocker tested. The binding present in the atopic dog was indistinct although the normal dog showed a few clear bands. However, goat serum was not superior to skimmed milk in terms of enhancing the clarity of bands in the normal or atopic dog. Since skimmed milk was cheap and easy to obtain, 1 % skimmed milk in TBS was therefore used as a blocker in subsequent immunoblotting experiments.

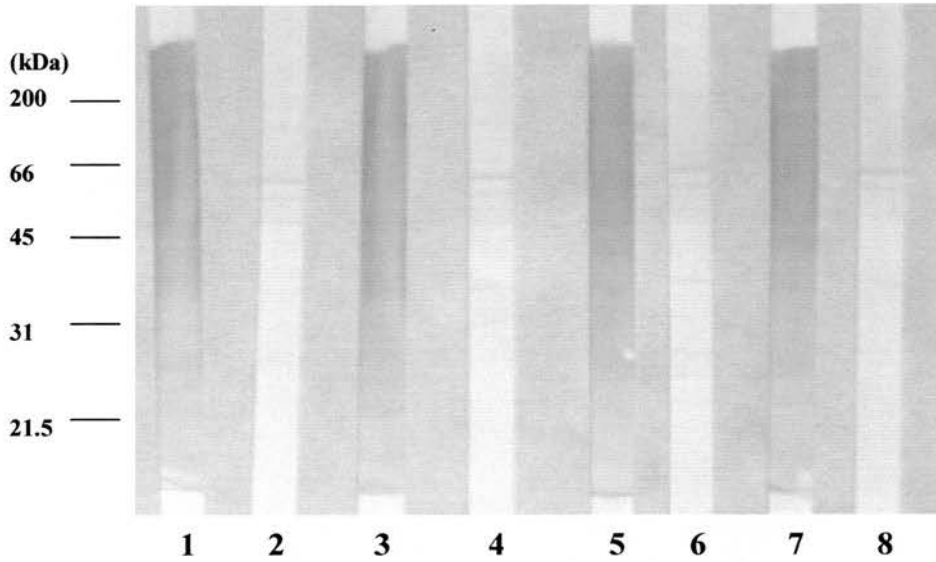


Figure 3.5 Evaluation of four different blocking reagents

Immunoblots showing the effects of different blotting reagents on different serum samples. Lanes 1-4 were blocked with skimmed milk in TBS and lanes 5-8, goat serum. The strips in lanes 1, 3, 5 and 7 were incubated with a serum sample from an atopic dog with *Malassezia* dermatitis, and the rest of the strips, a serum sample from a normal dog. There is a clear difference between the amount of staining shown by atopic dog serum and normal dog serum. However, no apparent difference was shown between strips blocked with goat serum and skimmed milk.

3.4 Optimisation of anti-canine IgG antibody dilution and substrate incubation time

Aims

With dog serum diluted to 1/100 and anti-dog IgG diluted to 1/5,000, the IgG binding was sub-optimal in the atopic dog with *Malassezia* dermatitis as shown in the previous experiment. The aim of these experiments was to determine the optimal dilution of anti-dog IgG with a serum dilution of 1/100. In addition, three different substrate incubation times were evaluated.

Methods

Twenty four pre-wetted strips containing separated *Malassezia* proteins were divided into three groups and placed into three 8-channel incubation trays. They were blocked with 1 % skimmed milk for 1 hour and washed with TTBS for 15 minutes. Half of the strips were incubated for 30 minutes with a serum sample from an atopic dog with *Malassezia* dermatitis and the other half with a serum sample from a normal dog, both of which were diluted 1/100 in dilution buffer. After further washing in TTBS for 15 minutes, the strips were incubated for 1 hour with anti-dog IgG diluted 1/5,000, 1/10,000, 1/20,000 or 1/50,000. Each dilution group included strips that had been incubated with sera from both the atopic and normal dog (see Figure 3.6 for experimental design). The strips were then washed in TTBS for a further 15 minutes, and developed with DAB peroxidase substrate. Three different incubation times were evaluated: the strips in trays 1, 2, and 3 were incubated with the substrate for 1, 15, and 25 minutes, respectively.

Figure 3.6 Experimental design

Strips	1	2	3	4	5	6	7	8
Blocking	1 % skimmed milk in TBS							
Serum	AM 1/100	N 1/100	AM 1/100	N 1/100	AM 1/100	N 1/100	AM 1/100	N 1/100
Anti-IgG	1/50,000		1/20,000		1/10,000		1/5,000	
DAB Tray 1	1 minute							
Tray 2	15 minutes							
Tray 3	25 minutes							

AM: atopic dog with *Malassezia* dermatitis; N: normal dog.

Results

Anti-dog IgG at dilutions of 1/20,000 and 1/50,000 did not generate sufficient staining, regardless of the incubation time of the DAB peroxidase substrate (Figure 3.7). The binding that developed under these conditions was very faint using the atopic dog serum compared to the strips incubated with anti-dog IgG diluted 1/10,000 or 1/5,000. No bands were detected with the normal dog serum with the higher dilutions of anti-dog IgG, whereas a few faint bands were visible when the anti-dog IgG was used at dilution of 1/10,000 or 1/5,000. The intensity of binding in the strips incubated with higher concentrations of anti-dog IgG did not differ noticeably between groups developed with DAB peroxidase substrate for different periods of time. However, anti-dog IgG at a dilution of 1/5,000 tended to produce a darker background, and this was more noticeable with the normal dog serum. The best results were obtained using an anti-dog IgG dilution of 1/10,000 and a 1-minute development time. These conditions were therefore used in subsequent experiments.

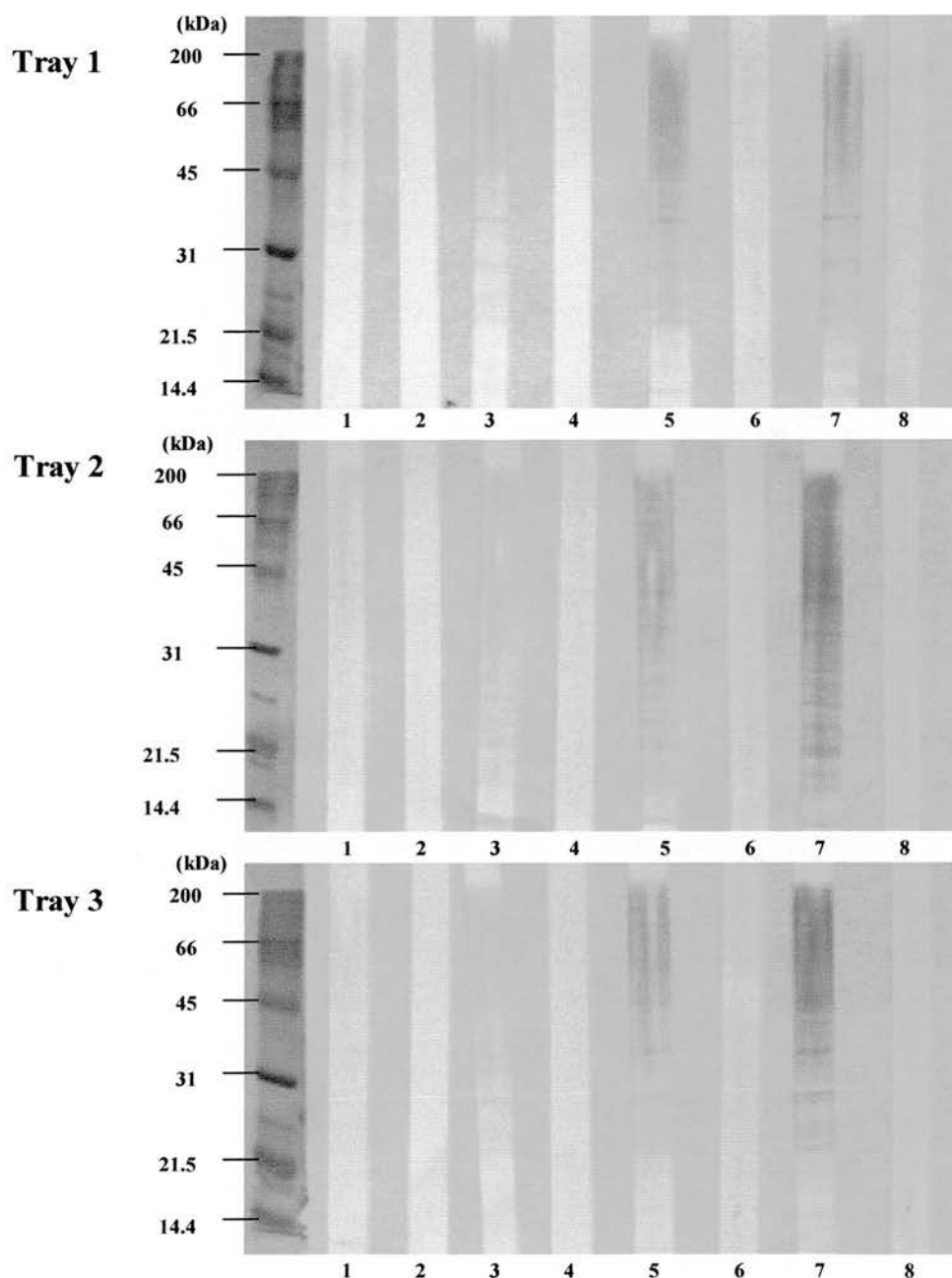


Figure 3.7 Optimisation of anti-dog IgG concentration and DAB peroxidase substrate development

Four different concentrations of anti-dog IgG, including 1/50,000, 1/20,000, 1/10,000 and 1/5,000, were evaluated. Each dilution group of anti-dog IgG comprised a serum sample chosen from an atopic dog with *Malassezia dermatitis* and a normal dog (see Figure 3.6 for experimental design). The strips in trays 1, 2, and 3 were developed with DAB peroxidase substrate for 1, 15, and 25 minutes, respectively. Comparing the results from the three trays, anti-dog IgG diluted 1/10,000 and a 1-minute substrate development were deemed to provide the best outcome (strips 5 and 6 in tray 1) and therefore used to analyse all subsequent serum samples.

IgG RESPONSE TO MALASSEZIA PACHYDERMATIS ANTIGENS IN DOGS

3.5 Detection of IgG binding proteins from *Malassezia pachydermatis* in atopic dogs with *Malassezia dermatitis*, atopic dogs without *Malassezia dermatitis* and normal dogs

Aims

In this section, the optimal conditions for immunoblotting to detect *Malassezia pachydermatis* specific IgG determined from earlier sections in this chapter were used to analyse individual dog serum samples from three groups of dogs: atopic dogs with *Malassezia dermatitis*, atopic dogs without *Malassezia dermatitis* and normal dogs.

Methods

The strips containing separated *Malassezia* proteins were pre-wetted with methanol and blocked for 1 hour with 1 % skimmed milk in TBS. After washing with TTBS for 15 minutes (3 × 5 minute rinses), each strip was incubated with 10 µl of dog serum diluted to 1 ml in dilution buffer (1 % skimmed milk in TTBS) for 30 minutes. The strips were then washed again in TTBS for a further 15 minutes. Bound IgG was detected by incubating the strips for 1 hour with horseradish peroxidase conjugated goat anti-dog IgG heavy and light chain diluted 1/10,000 in dilution buffer. After further washing with TTBS for 15 minutes, the strips were developed with DAB peroxidase substrate for about 1 minute. The molecular weights of IgG binding proteins were determined using standard curves generated by analysing the position of molecular weight markers on Coomassie blue stained membranes.

Results

The IgG immunoblots of individual dog sera from the three different groups are shown in Figure 3.8 A-C. The amount of staining seen in the normal dogs (Figure 3.8 A) was much weaker than that seen in the atopic dogs with cytological evidence of *Malassezia* overgrowth (Figure 3.8 B). The binding pattern seen in atopic dogs without *Malassezia* dermatitis lay between the previous two groups (Figure 3.8 C).

More than half of the normal dogs showed multiple indistinct bands between 42 and 68 kDa, but clearer bands could be seen at 42 (in 83 % of dogs), 45 (61%), and 82 kDa (67 %) (Figure 3.9 A). Two dogs showed relatively strong binding patterns (No. 9 and 18) and one dog had a prominent band at 27 kDa (No. 15).

In the atopic dogs with *Malassezia* dermatitis, there was more intense staining of indistinct bands between 45 and 51 kDa. However, distinct IgG-binding proteins with molecular weights of 25 (78 %), 29 (67 %), 42 (94 %), 61 (67 %), 82 (89 %), and 90 kDa (61 %) were recognised by more than 50 % of the atopic dogs with *Malassezia* dermatitis (Figure 3.9 B). Some atopic dogs also showed binding to various low molecular weight proteins between 14 and 42 kDa. Antigens of 28-30 (55 %), 42 (95 %), 45-51 (80 %), 61 (50 %), 82 (65 %) and 90 (55 %) kDa were also recognised by more than 50 % of the sera from atopic dogs without *Malassezia* dermatitis (Figure 3.9 C).

The blots of the three groups demonstrate that most dogs show IgG binding to multiple *Malassezia* antigens. However, atopic dogs with *Malassezia* dermatitis had a much greater IgG response than the normal dogs, and the majority showed binding to a protein of 25 kDa which was not seen in most dogs of the other two groups.

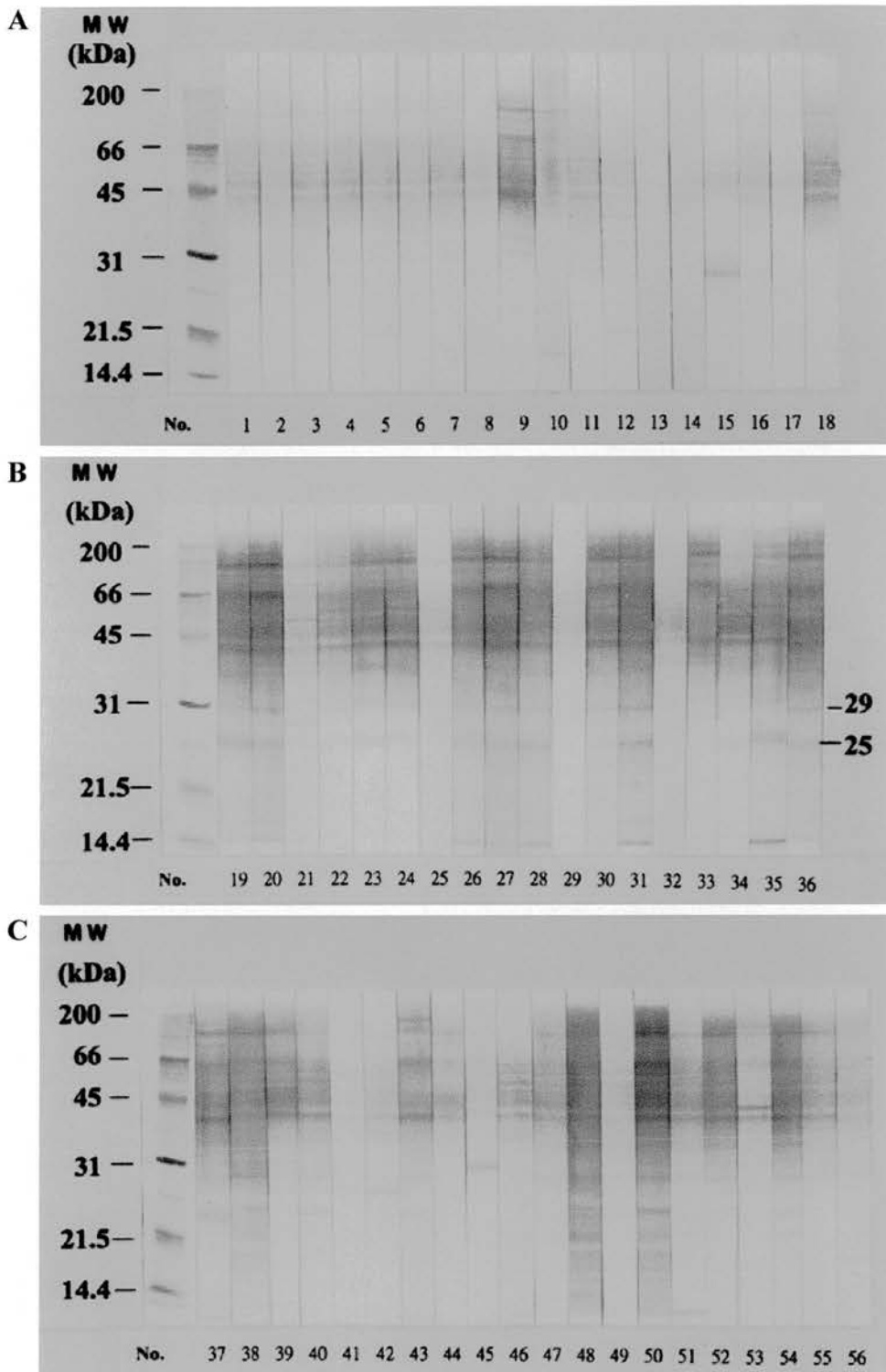


Figure 3.8 A-C IgG-binding components in extracts of *M. pachydermatis* detected by immunoblotting with dog sera from 3 different groups

A: Normal dogs; B: Atopic dogs with *Malassezia* dermatitis and C: Atopic dogs without *Malassezia* dermatitis. The numbers along the bottom signify the strips probed with individual dog sera. The molecular weight standards on the left are 200, 66, 45, 31, 21.5, and 14.4 kDa.

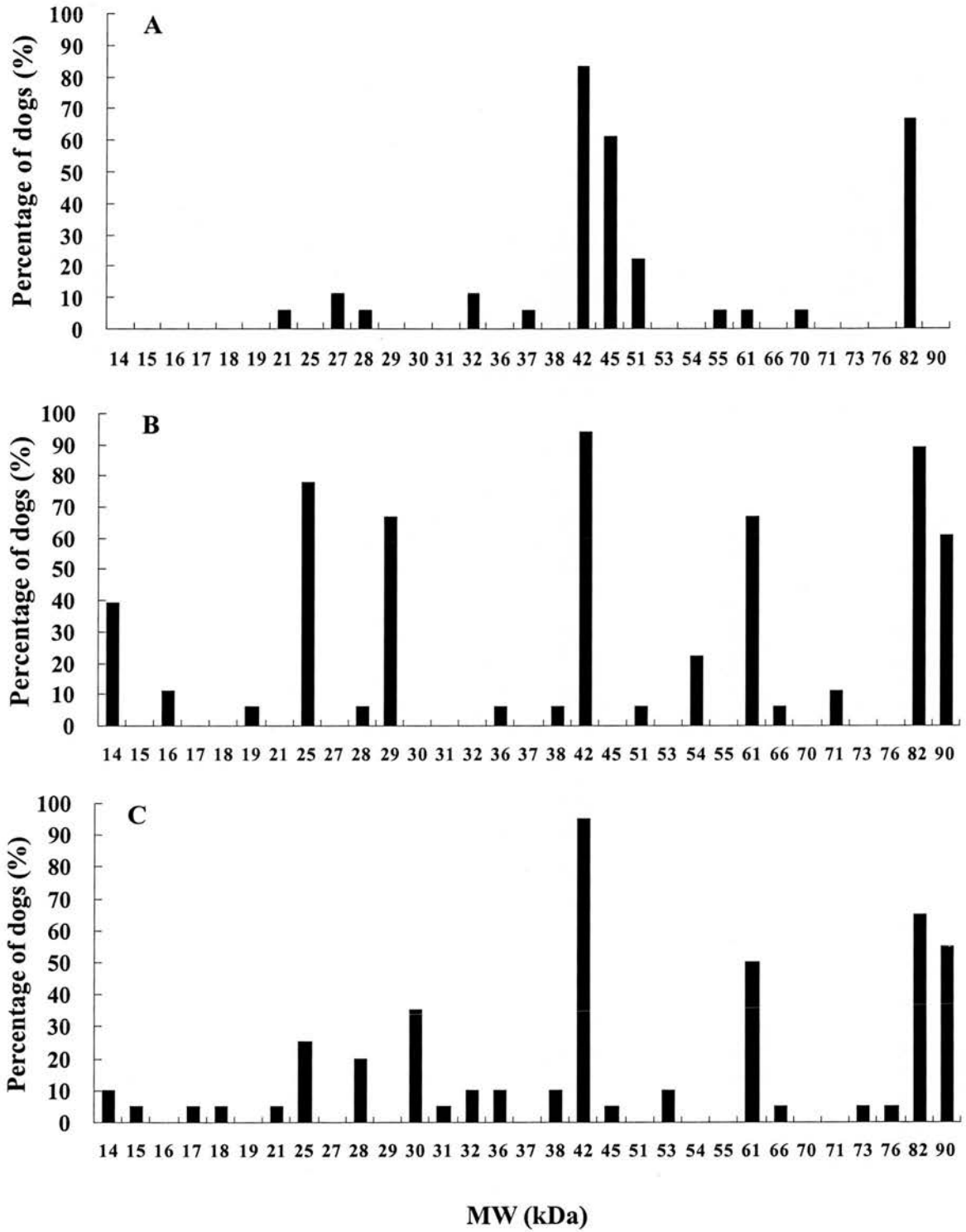


Figure 3.9 A-C Percentage of dogs from three different groups showing IgG binding to individual antigenic components in extracts of *M. pachydermatis*

A: Normal dogs; B: Atopic dogs with *Malassezia* dermatitis and C: Atopic dogs without *Malassezia* dermatitis. X axis: molecular weights of the bands detected on the strips; Y axis: percentage of dogs reacting to those bands.

3.6 Discussion

In this chapter, specific IgG binding antigens in extracts of *Malassezia pachydermatis* were demonstrated with sera of atopic dogs with *Malassezia* dermatitis, atopic dogs without *Malassezia* dermatitis and normal dogs using western blotting. The results of the immunoblotting with individual dog sera indicate that most dogs have an IgG response to multiple antigens of *M. pachydermatis*.

Approximately half of the normal dogs showed faint IgG binding to multiple indistinct bands between 42-68 kDa, but clearer bands could be seen at 42, 45, and 82 kDa. These difficulties in distinguishing bands could be overcome using two-dimensional SDS-PAGE to further separate the antigens by their isoelectric points and immunoblotting (Onishi *et al.* 1999). Nevertheless, our results suggest that the commensal population of *M. pachydermatis* is recognised by the dog's immune system leading to an antibody response. This is in agreement with studies in man, which showed that *Malassezia*-specific IgG was detectable in healthy individuals by ELISA (Tengvall Linder *et al.* 2000; Savolainen *et al.* 2001). Whether or not this IgG response seen in normal dogs is protective in any way remains to be determined.

The degree and intensity of staining on the blots from the atopic dogs with *Malassezia* dermatitis was much greater than that seen using sera from the normal dogs, indicating that the majority of atopic dogs with *Malassezia* dermatitis had a greater IgG response to *M. pachydermatis*. This result is in accordance with a recent finding that dogs with skin disease associated with *M. pachydermatis* develop high serum titers of *M. pachydermatis*-specific IgG (Bond *et al.* 1998). Furthermore, more numerous IgG-binding proteins were observed on the blots from the atopic dogs with

Malassezia dermatitis than using normal sera. Some of these proteins were the same in both groups (molecular weights of 42, 45-51, 82 kDa). However, an antigen of 25 kDa was recognised by the majority of atopic dogs with *Malassezia dermatitis* but was not recognised by most dogs in the other two groups. At this stage, the identity of this protein and its function are not known. Likewise, the significance of the IgG response to this antigen in the atopic dogs is unknown. It is known that IgG is required to opsonise staphylococci for phagocytosis by neutrophils (Shearer & Day 1997b). Similarly, the IgG may act as an opsonin to facilitate phagocytosis of *Malassezia* antigens. This could have a protective function but the IgG could also activate the complement system and exacerbate the underlying inflammation. It has been shown that *Pityrosporum orbiculare* and *P. ovale* are capable of activating the classical and alternative complement pathways (Belew *et al.* 1980; Sohnle & Collins-Lech 1983). It has also been suggested that zymogen in the cell wall of *M. pachydermatis* might activate complement leading to epidermal damage and inflammation (Mason 1993). Despite the uncertainty surrounding the role of this antigen, the IgG response seen in the atopic dogs with *Malassezia* overgrowth but not in the normal dogs suggests that it may have some clinical relevance in the disease.

IgG-binding components in extracts of *M. pachydermatis* have also been investigated with sera of healthy dogs and non-atopic dogs with *Malassezia dermatitis* (Bond & Lloyd 2002). Proteins of 132, 66 and 50-54 kDa were recognised by most sera, but affected dogs showed immunoreactivity mainly to proteins of 219, 110, 71, and 42 kDa (Bond & Lloyd 2002). Comparing the relative positions of bands and molecular weight standards, we infer that some of these results are likely to represent the same

bands as those seen in this study. However, the 25 kDa antigen was not recognised by sera from the non-atopic dogs with *Malassezia* dermatitis, again suggesting a degree of specificity for this response in dogs with atopic dermatitis.

The pattern of IgG binding revealed by sera from the atopic dogs without *Malassezia* dermatitis lies between that revealed by sera from the normal dogs and atopic dogs with *Malassezia* dermatitis. Most of these dogs also recognised antigens of 28-30, 42, 45-51, 61, 82, and 90 kDa. Due to the difficulty of precisely resolving the molecular weight of proteins on SDS-PAGE gels, it is possible that the bands detected at 28 and 30 kDa are the same as the 29 kDa antigen seen in the group with *Malassezia* overgrowth. There are some possible explanations for this intermediate pattern. White *et al.* (1996) compared the carriage of *M. pachydermatis* on clinically normal skin of the intradermal test site in atopic dogs and trunkal glabrous skin of healthy dogs. They found that atopic dogs had significantly higher yeast culture scores than healthy dogs. Hence, the atopic dogs without *Malassezia* dermatitis could still have had elevated populations of the yeast without having cytological evidence of overgrowth. Also, some of the atopic dogs may have had *Malassezia* dermatitis or otitis in the past, leading to a residual IgG response. Finally, the immune system of atopic dogs may be sensitized to the normal population of the commensal through their frequent scratching behavior. It has been suggested that disruption of stratum corneum barrier function by scratching may enhance exposure of the skin immune system to yeast allergens (Morris *et al.* 1998). However, the 25 kDa antigen was only seen in less than 25 % of the atopic dogs without *Malassezia* overgrowth, suggesting that an IgG response to this protein is clinically relevant in the disease.

In contrast to two human studies showing no difference in levels of *Malassezia*-specific IgG between patients with atopic dermatitis and healthy controls (Tengvall Linder *et al.* 2000; Savolainen *et al.* 2001), the results of this chapter demonstrated that atopic dogs generally developed a greater IgG response to *M. pachydermatis* than normal dogs, with a higher frequency in atopic dogs with *Malassezia* dermatitis. This could indicate that people and dogs with atopic dermatitis might react differently to *Malassezia* organisms colonising on their skin. Tengvall Linder *et al.* (2000) also showed that the *Malassezia*-specific serum IgG levels did not correlate with the patch test responses to *Malassezia* extracts in patients with atopic dermatitis. Since intradermal skin testing to *Malassezia* extracts was not performed in dogs in the present study, no comparison can be made between human beings and dogs in this respect. Whether the greater IgG response to *M. pachydermatis* seen in the atopic dogs simply reflects higher exposure to the organism, or plays a role in the pathogenesis of atopic dermatitis is uncertain.

In summary, the experiments in this chapter have identified a number of IgG binding proteins in *M. pachydermatis*. The IgG response was greater in atopic dogs with *Malassezia* dermatitis and an antigen of 25 kDa appeared to be the most important. In order to determine the identity of the 25 kDa antigen, the crude *Malassezia* extracts could be purified using high-performance liquid chromatography (HPLC) and separated by electrophoresis. The 25 kDa antigen recognised by the sera of atopic dogs with *Malassezia* dermatitis could then be identified by N-terminal sequencing. It has been suggested that T-cell mediated immune responses are important in immunity against superficial mycoses (Hay 1992). The purified antigen could potentially be used to study cell-mediated immune responses to *M.*

pachydermatis in the future, which would further elucidate the antigen recognition and cellular responses in atopic dogs with *Malassezia* dermatitis. The cellular location of this 25 kDa antigen is unknown and awaits further investigation. The *Malassezia* extract used in this study was produced by grinding the organisms with glass beads and therefore consisted of fragmented cell walls and various intracellular components. The 25 kDa antigen could possibly be intracellular, on the cell wall, or both. Determination of its location and identity may help us further understand the mechanisms by which *M. pachydermatis* triggers immune responses in the host.

Chapter 4

IDENTIFICATION OF MAJOR ALLERGENS OF *MALASSEZIA PACHYDERMATIS* IN DOGS WITH ATOPIC DERMATITIS AND *MALASSEZIA* OVERGROWTH

4.1 Introduction

Atopic diseases represent clinical presentations of Type I hypersensitivity reactions that are generally triggered by an increased production of IgE in response to environmental allergens (Platts-Mills 2001). Exposure to antigens leads to production of antigen-specific IgE that binds to mast cells, resulting in the individual being sensitised. Subsequent re-encounter with the allergens cross-links mast cell bound IgE, bringing about mast cell degranulation, release of inflammatory mediators and clinical signs of atopic diseases (Platts-Mills 2001). The association between atopic diseases and IgE concentrations, both total and allergen-specific, has therefore been investigated extensively.

4.1.1 Total serum IgE in patients with atopic diseases

Total serum IgE levels are often raised in human patients with atopic diseases such as allergic rhinitis, asthma and atopic dermatitis (O'Loughlin *et al.* 1977; Zetterstrom & Johansson 1981; Platts-Mills 2001). It has also been found that there is a positive correlation between the severity of atopic dermatitis and increased serum IgE levels in atopic individuals (Clendenning *et al.* 1973). Determination of total serum IgE concentrations has been used to provide additional evidence for diagnosis of suspected atopic diseases, especially in children, in which elevated IgE levels can also be helpful to predict subsequent development of atopic diseases (Kjellman 1976;

Lindberg & Arroyave 1986; Fifield *et al.* 1987).

Studies comparing total serum IgE concentrations in atopic and healthy dogs reveal different results to those found in people. No significant differences were found between the two groups when measured by radial immunodiffusion techniques or ELISA (Vriesendorp *et al.* 1975; Schwartzman 1984; Nimmo Wilkie *et al.* 1990; Hill *et al.* 1995; Jackson *et al.* 2002). Also, the severity of the clinical signs in atopic dogs did not correlate with total serum IgE concentrations (Jackson *et al.* 2002). It has been speculated that the discrepancy between the two species is due to a greater exposure of the canine population to parasite infestation (Vriesendorp *et al.* 1975; Hill *et al.* 1995), as elevated IgE levels are also seen in endoparasitic diseases (Heiner & Rose 1970; Merrett *et al.* 1976). Furthermore, total serum IgE concentrations in puppies has little value in predicting the onset of atopic dermatitis (DeBoer & Hill 1999).

4.1.2 IgE response to environmental allergens

Various techniques have been used in the diagnosis and investigation of atopic dermatitis in man and dogs to detect IgE-mediated immunological responses toward environmental antigens, mainly *in vivo* skin tests (e.g. skin prick test (SPT) and intradermal test (IDT)), *in vitro* antibody detection assays (e.g. ELISA and radioallergosorbent test (RAST)), and antigen identification techniques, such as Western immunoblotting .

The aim of skin testing is to detect allergen-specific IgE antibodies on the surface of cutaneous mast cells, which cross-link in the presence of allergens triggering mast

cell degranulation. The release of histamine as well as other mediators then causes vasodilatation and increased vascular permeability leading to tissue oedema and the development of a wheal (Frew 1997). In humans, skin testing is usually performed by the skin prick method using diluted antigen extracts because the IDT involves increased discomfort and risk of systemic reactions (Demoly *et al.* 1998). In contrast, IDT is widely and almost exclusively used in dogs to define the allergens that animals are hypersensitive to and a variety of environmental antigens are usually included in the antigen panel (Hillier & DeBoer 2001). The antigens commonly used in IDTs generally fall into the following categories: mite antigens; epithelial antigens; pollens from grass, trees and weeds; mould antigens; and insect antigens (reviewed in Hill & DeBoer 2001). By comparing the data published, the importance of environmental antigens in canine atopic dermatitis seems to be associated with geographical locations, with dust mite and epidermal antigens being important in both the USA and Europe whereas pollen and mould antigens are more important in the USA than that in Europe (Hill & DeBoer 2001).

In vitro tests measuring circulating antigen-specific IgE in sera are frequently used to assist diagnosis in atopic human patients (Merrett 1997). For the diagnosis of canine atopy, although varying correlations between IDT and *in vitro* tests have been reported (Halliwell & Kunkle 1978; Codner & Lessard 1993; Bond *et al.* 1994b), *in vitro* tests are generally considered to be less valuable than the IDT because they only evaluate one aspect of the IgE/mast cell/microvasculature axis. There can also be problems of reproducibility, sensitivity, specificity and false positive or negative results with the assays (Codner & Lessard 1993; Bond *et al.* 1994b; Plant 1994; DeBoer & Hillier 2001). As with the use of *in vivo* and *in vitro* tests in man, the

results of these tests in dogs should be interpreted in conjunction with the animals' clinical history and signs (Kleinbeck 1994; Scott *et al.* 2001c).

The use of western immunoblotting techniques allows major allergens to be determined from crude antigen extracts. These can then be further characterised by biochemical and molecular techniques. A wide range of purified allergens have been determined in man and many of them have now been identified (Chua *et al.* 1988; Nilsen & Paulsen 1990; Thakur 1991; Lin *et al.* 1994; Asturias *et al.* 1997; Okada *et al.* 1998; Suphioglu *et al.* 1999; Vrtala *et al.* 1999; Mills *et al.* 1999; Su *et al.* 1999). Similar studies are limited in dogs and have predominantly focused on *Dermatophagoides* mites (Noli *et al.* 1996; Masuda *et al.* 1999; McCall *et al.* 2001; Nuttall *et al.* 2001).

4.1.3 Allergen-specific IgE responses to cutaneous microorganisms

Due to the increased susceptibility of the skin of patients with atopic dermatitis (man and dogs) to colonisation with *Staphylococcus* and *Malassezia* species, many studies looking at the IgE responses to these microorganisms have been undertaken in man. In contrast, only a few investigations of this kind have been performed in dogs.

4.1.3.1 IgE responses to *Staphylococcus* bacteria

The results of numerous studies have suggested that specific IgE antibodies against *Staphylococcus aureus* may play an important role in human atopic dermatitis. It has been found that some human patients with atopic dermatitis and cutaneous staphylococcal infections have detectable IgE to *Staphylococcus aureus* in their sera, using an immunoradiometric method to detect specific IgE against whole organisms

or purified cell wall (Friedman *et al.* 1985). Studies on IgE responses to a variety of exotoxins secreted by *Staphylococcus aureus* showed that the majority of patients suffering from atopic dermatitis had specific IgE antibodies to enterotoxins A and B (Leung *et al.* 1993; Tada *et al.* 1996) and toxic shock syndrome toxin-1 (TSST-1) (Leung *et al.* 1993) as measured by ELISA. The severity of atopic dermatitis has been reported to correlate with the levels of IgE antibodies to enterotoxins A and/or B (Bunikowski *et al.* 1999) or enterotoxin B alone (Nomura *et al.* 1999; Breuer *et al.* 2000). In one study, TSST-1 was demonstrated to augment allergen-specific IgE production (Hofer *et al.* 1999). Stimulation of IgE synthesis by peripheral blood mononuclear cells (PBMC) *in vitro* has also been shown with two surface proteins of *S. aureus*, NP-tase and p70, in patients with atopic dermatitis (Jahreis *et al.* 2000).

The role of *Staphylococcus intermedius* and its exotoxins in the pathogenesis of canine atopic dermatitis has not been fully defined. It has been demonstrated that dogs with recurrent pyoderma, either idiopathic or associated with atopy, have higher levels of anti-staphylococcal IgE in their sera than healthy dogs, as measured by ELISA techniques (Halliwell 1987; Morales *et al.* 1994). However, the specific IgE responses to staphylococcal exotoxins or other staphylococcal components have not yet been studied in dogs.

4.1.3.2 IgE responses to *Malassezia* yeasts

The IgE responses to *Malassezia* species on human skin have been studied extensively and various methods have been used to detect *Malassezia*-specific IgE, including IDT, SPT, histamine release tests and *in vitro* serological tests such as ELISA, RAST and immunoblotting. Although direct comparisons between the

results of different studies are difficult due to the discrepancies in the methodologies used by different investigators (such as the source of allergens and the definition of positive reactions), similar findings have been documented using intradermal and skin prick tests with *Malassezia* antigens. An increased sensitivity to these tests has been reported in patients with generalised atopic dermatitis (Young *et al.* 1989; Rokugo *et al.* 1990; Broberg *et al.* 1992) or those with lesions predominantly in the head and neck (Waersted & Hjorth 1985; Kieffer *et al.* 1990; Nissen *et al.* 1998). Using basophil histamine release test with whole blood samples, it has also been found that most patients with atopic dermatitis had positive responses to *Malassezia* extracts with a higher percentage seen in patients with head and neck dermatitis (Kieffer *et al.* 1990; Nissen *et al.* 1998). Increased concentrations of *Malassezia*-specific IgE were also found in the sera of patients with atopic dermatitis compared to those with other atopic diseases or healthy controls, as measured by either ELISA (Wessels *et al.* 1991) or RAST (Young *et al.* 1989; Nordvall *et al.* 1992; Savolainen *et al.* 2001). Also, these antibodies were found to be present more frequently in the first group (Nordvall & Johansson 1990; Broberg *et al.* 1992).

In contrast to the correlation between the concentration of specific IgE to certain exotoxins derived from *Staphylococcus aureus* and the severity of atopic dermatitis in human patients, a similar correlation has not been found with *Malassezia*-specific IgE (Wessels *et al.* 1991; Back *et al.* 1995). However, by using western immunoblotting to detect *Malassezia*-specific IgE, and the criteria that more than 50 % of the patients' sera reacting represents major allergens, a number of major IgE-binding proteins in the range of 9-110 kDa have now been documented in man (Johansson & Karlstrom 1991; Jensen-Jarolim *et al.* 1992; Zargari *et al.* 1994; Lintu

et al. 1997; Nissen *et al.* 1998). Some antigens defined as major allergens in one study have been cited as minor allergens by other investigators (listed in Ashbee & Evans 2002). This is likely to be due to the disparity between methodologies and antigen preparations used in different studies. Of the numerous antigens documented, a limited number of antigens have been further characterised. Nine allergens of *Malassezia furfur* (Mal f 1- Mal f 9) were recently sequenced and expressed as recombinant proteins (Schmidt *et al.* 1997; Yasueda *et al.* 1998; Onishi *et al.* 1999; Lindborg *et al.* 1999; Rasool *et al.* 2000). However, the strain used in some of these studies has now been re-assigned to the species *M. sympodialis* (Scheynius *et al.* 2002), and the current nomenclature of purified *Malassezia* allergens is summarised in Table 4.1.

Table 4.1 Three allergens of *Malassezia furfur* and six allergens of *Malassezia sympodialis* that have been sequenced and expressed as recombinant proteins

Allergen name	MW (kDa)	Percentage of AD patients reacting	Identity
Mal s 1	37	70	No homology to known proteins Membrane or secreted cell wall protein
Mal f 2	21	72	Homology to peroxisomal membrane proteins of <i>Candida boidinii</i> and <i>Aspergillus fumigatus</i> (Asp f 3)
Mal f 3	20	70	Homology to peroxisomal membrane proteins of <i>Candida boidinii</i> and <i>Aspergillus fumigatus</i> (Asp f 3)
Mal f 4	35	83	Showing 57 % homology to mitochondria malate dehydrogenase from <i>Saccharomyces cerevisiae</i>
Mal s 5	18.2	48	No homology to known proteins
Mal s 6	17.2	48	Showing 82 % homology to cyclophilin from <i>Schizosaccharomyces pombe</i>
Mal s 7	16.2	40	No homology to known proteins
Mal s 8	19.2	40	No homology to known proteins
Mal s 9	14	24	No homology to known proteins

AD: atopic dermatitis

It is important to note that many of the studies described above were performed using the old classification of *Malassezia* species, and therefore might reflect the characteristics of other more recently described *Malassezia* species. A study

investigating IgE binding components in five of the seven species currently classified under the *Malassezia* genus, including *Malassezia furfur*, *M. globosa*, *M. restricta*, *M. slooffiae*, and *M. sympodialis*, showed that both species-specific and common antigenic components were present between species, and the molecular weights of bands most frequently recognised by the sera of AD patients were 67-72 (28 %), 45-50 (80 %), 35-40 (39 %), 43-46 (20 %) and 19-22 kDa (43 %), respectively (Koyama *et al.* 2001). It is likely that further studies comparing different species, and the use of molecular techniques, will define more precisely the identity of major allergens of *Malassezia* species.

In contrast to the large number of studies that have been carried out in humans during the last two decades, it is only recently that IgE responses to *Malassezia pachydermatis* in dogs have been investigated. Immediate hypersensitivity responses to intradermal injections of *M. pachydermatis* extracts have been demonstrated in atopic dogs with *Malassezia* overgrowth (Morris *et al.* 1998; Bond *et al.* 2002a), suggesting that allergens from the organisms may be involved in the pathogenesis of canine atopic dermatitis. Atopic dogs with and without *Malassezia* dermatitis and /or otitis have also been shown to have significantly higher levels of *Malassezia*-specific IgE than either healthy dogs or non-atopic dogs with *Malassezia* dermatitis and /or otitis as detected using ELISA (Nuttall & Halliwell 2001). However, the IgE binding epitopes of *Malassezia pachydermatis* have not yet been investigated.

4.1.4 Aims

The aim of this section was to complement the IgG data in chapter 3 by further characterising the humoral immune response to *Malassezia pachydermatis* by

comparing IgE responses to separated antigens of the yeast in atopic dogs with *Malassezia* dermatitis and normal dogs. The initial objective of the experiments was to develop and validate a Western blotting technique to detect specific IgE binding proteins from *M. pachydermatis*. The first part of this chapter describes six protocols that were investigated, and the second part describes the detection of IgE binding allergens of *M. pachydermatis* in the two groups of dogs.

**VALIDATION AND OPTIMISATION OF METHODOLOGY TO DETECT
IgE BINDING PROTEINS IN *M. PACHYDERMATIS***

4.2 Summary of methodologies and reagents evaluated

Various methodologies and reagents were evaluated to detect IgE binding proteins in *M. pachydermatis* (summarised in Table 4.2). Two methods were investigated to allow immunoblot membranes to be probed with individual dog sera: cutting the membrane into narrow strips or placing the whole membrane into a multi-channel miniblotted (Immunitics Inc. Cambridge, USA). The blocking reagents that were evaluated included skimmed milk (1-10 %), casein solution (Vector Laboratories, Inc., Burlingame, CA, USA), Tween 20 (Fisher Scientific, UK), Tween 80 (Sigma, UK), and an avidin/biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA, USA). The following IgE specific reagents were evaluated: a polyclonal epsilon chain specific goat anti-dog IgE conjugated with horseradish peroxidase (HRP) (Bethyl Laboratories, Montgomery TX, USA); two monoclonal mouse anti-dog IgE antibodies (D9#3116, kindly donated by Dr. D.J. DeBoer, University of Wisconsin, USA and E6-71A1, Custom Monoclonals International, W. Sacramento, USA); and a biotinylated recombinant human IgE receptor alpha chain (kindly donated by Dr. C. McCall, Heska Corporation, Fort Collins, USA). For visualization of binding, the following conjugates labeled with either alkaline phosphatase (AP) or HRP were used: a monoclonal mouse anti-goat IgG-AP (Sigma, UK); a monoclonal bovine anti-mouse IgG1-HRP (Serotec, UK); two polyclonal goat anti-mouse IgG antibodies labeled either with HRP (Sigma, UK) or AP (Southern Biotechnology Associates, Inc., USA); and two avidin reagents (Vectastain[®] Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA and Extravidin[®]-peroxidase, Sigma, UK). Two different detection systems were evaluated: substrate colour change and chemiluminescence.

For standard blots, DAB (3, 3'-diaminobenzidin, Vector Laboratories, Inc., Burlingame, CA, USA) and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, Sigma, UK) were used to detect HRP and AP labeled reagents, respectively. For chemiluminescent blots, ECLTM (Amersham pharmacia biotech, UK) was used for HRP labeled reagents and CDP-StarTM (Tropix, Inc., Bedford, USA) for AP labeled reagents (Table 4.2). Each protocol will be described in detail in the following sections.

Table 4.2 Summary of methodologies and reagents evaluated in this study to detect IgE binding proteins in *M. pachydermatis*

PROTOCOL	REAGENTS				Comments*
	IgE-specific Reagent	Secondary- developing reagent	Substrate	Detection	
1	Goat anti-dog IgE-epsilon chain specific, HRP (Bethyl Laboratories)		DAB	Colour change on blots	Abandoned due to lack of sensitivity
2	Goat anti-dog IgE-epsilon chain specific, HRP (Bethyl Laboratories)	Monoclonal anti-goat IgG, AP (Sigma)	BCIP/NBT	Colour change on blots	Abandoned due to lack of sensitivity
3	Monoclonal mouse anti-dog IgE (Dr. D.J. DeBoer)	Goat anti-mouse IgG, HRP (Sigma)	ECL™	Chemiluminescence detected on X-ray films (Hyperfilm™ ECL™, Amersham Life Science)	Abandoned due to cross-reactivity of the secondary antibody with canine IgG
4	Monoclonal mouse anti-dog IgE (Dr. D.J. DeBoer)	Bovine anti-mouse IgG1, HRP (Serotec)	ECL™	Chemiluminescence detected on X-ray films	Abandoned due to cross-reactivity of the secondary antibody with canine IgG
5	Biotinylated recombinant human IgE receptor alpha chain (Heska)	Vectastain® Elite ABC kit (Vector Laboratories) Extravidin®-peroxidase (Sigma)	ECL™	Chemiluminescence detected on X-ray films	Abandoned due to cross-reactivity of the secondary-developing reagents with <i>Malassezia</i> extracts
6	Monoclonal mouse anti-dog IgE E6-71A1 (Custom Monoclonals International)	Goat anti-mouse IgG1, AP (Southern Biotechnology Associates) before and after passing through a canine IgG column three times	CDP-Star™	Chemiluminescence detected using Kodak Digital Science™ Image Station 440CF	Successful

HRP: horseradish peroxidase; AP: alkaline phosphatase;

DAB: 3, 3'-diaminobenzidine; BCIP/NBT: 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium

* See following sections for further details

Chromogenic methods

4.3 Protocol 1–detection of IgE binding proteins with an epsilon chain specific goat anti-canine IgE antibody conjugated with HRP

Aims

In chapter 3, IgG binding proteins from *Malassezia pachydermatis* were demonstrated using an anti-dog IgG antibody and chromogenic detection. In this section, the initial aim was to test the feasibility of using the same detection method with an anti-dog IgE antibody to detect specific IgE binding allergens in *M. pachydermatis* in atopic dogs with *Malassezia* dermatitis.

Methods

The SDS-PAGE and western blotting were performed as described in chapter 2. Briefly, the *Malassezia* extract prepared with glass beads (123 µg, 150 µl of 1/20 dilution) was mixed with reducing sample buffer and a final volume of 300 µl was added into a broad well running across the top of the gel. The electrophoresis was run at 200 V for 60 minutes and the proteins were transferred to a membrane. The membrane was then cut into narrow strips in order to be probed with individual sera or different reagents. The strips were dipped in methanol for a few seconds and rinsed with de-ionised water before placing into 8-channel trays.

Sixteen pre-wetted strips containing separated *Malassezia* proteins were placed into two 8-channel incubation trays. They were blocked with 1 % skimmed milk in TBS for 1 hour and washed with TBS containing 0.05 % Tween 20 (TTBS) for 15 minutes (3 x 5 minute rinses). Due to the relatively low concentration of IgE in dog serum compared to IgG, the strips were then incubated with dog sera for 1 hour rather than

30 minutes as in the IgG study to increase the probability of binding and to enhance the signal. Lower dilutions of dog sera and anti-IgE antibody were also used to increase sensitivity. The first two strips in each tray were incubated with dilution buffer (1 % skimmed milk in TTBS) alone to act as controls (Figure 4.1). The rest of the strips were divided into two groups. Half of the strips were incubated with a serum sample from an atopic dog with *Malassezia* dermatitis and the other half with a serum sample from a normal dog, both of which were diluted 1/5, 1/10 or 1/50 in dilution buffer. After further washing in TTBS for 15 minutes, the strips were incubated for 1 hour with a HRP conjugated goat anti-dog IgE epsilon chain (Bethyl Laboratories, Montgomery TX, USA) diluted 1/100 or 1/500. The strips were then washed in TTBS for a further 15 minutes, and developed with DAB peroxidase substrate.

Figure 4.1 Experimental design

Strips	1	2	3	4	5	6	7	8
Blocking	1 % skimmed milk in TBS							
Serum	Buffer	Buffer	AM 1/50	N 1/50	AM 1/10	N 1/10	AM 1/5	N 1/5
Anti-IgE	Tray 1			1/500				
	Tray 2			1/100				

AM: atopic dog with *Malassezia* dermatitis; N: normal dog.

Results

No visible bands were seen in any of the groups, regardless of the dilutions of dog sera or anti-dog IgE (Figure 4.2). The strips incubated with anti-dog IgE diluted 1/500 generated light brown background, but, no bands were visible in any of the strips. Similar results were seen in strips incubated with anti-dog IgE diluted 1/100. In addition to the lack of bands, these strips also showed darker background

compared to those in tray 1 due to the lower dilution of anti-dog IgE. Despite the low dilutions of both dog sera and anti-dog IgE, no specific binding was generated using this protocol (lanes 7 and 8 in tray 2), indicating that it was not sensitive enough to detect *Malassezia*-specific IgE. Further optimisation was therefore required to increase the sensitivity.

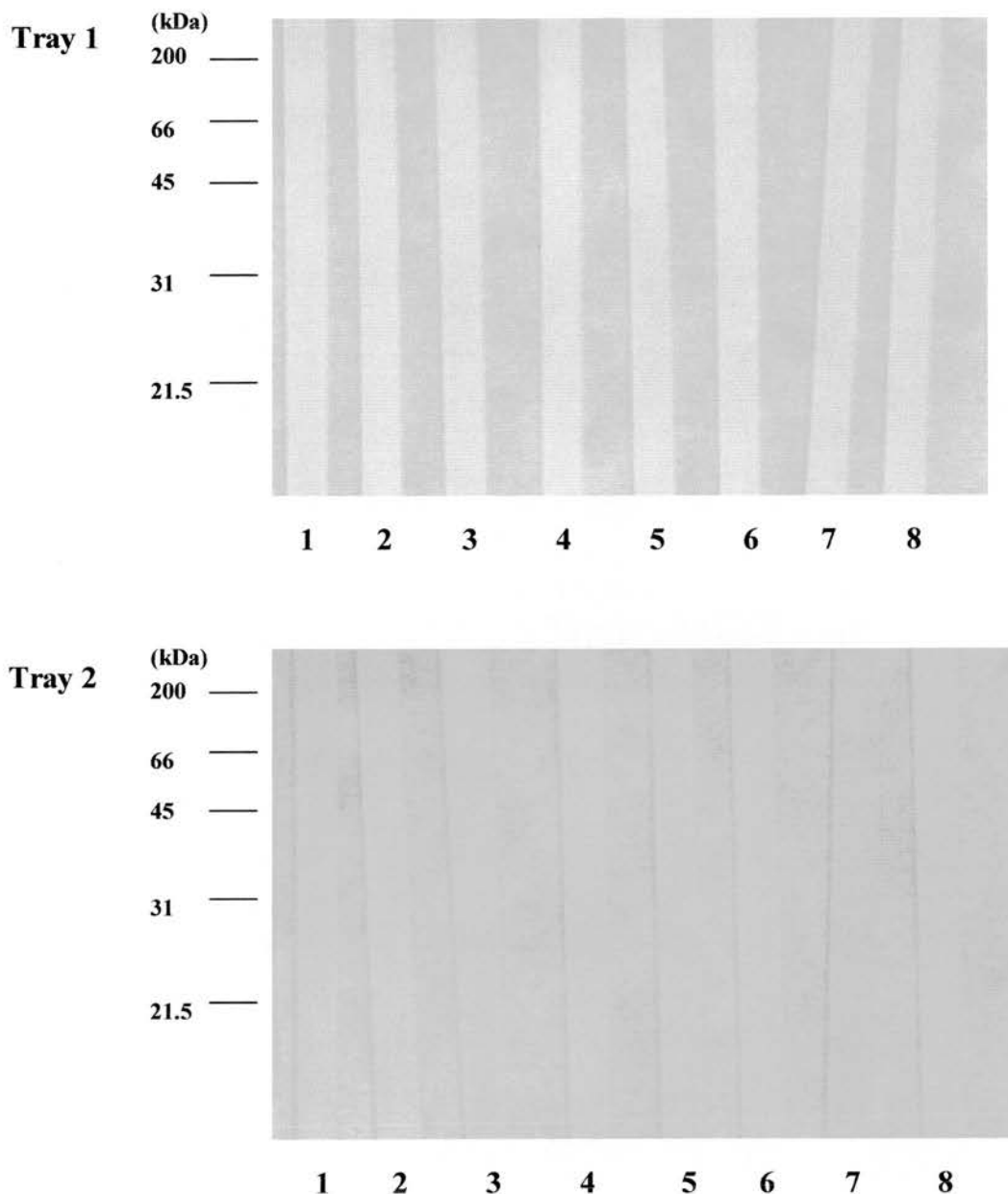


Figure 4.2 Examination of the feasibility of using a horseradish peroxidase conjugated goat anti-dog IgE to detect IgE binding proteins from *Malassezia pachydermatis*

The strips in lanes 1 and 2 were incubated with dilution buffer alone and the rest were incubated with dog sera diluted 1/50, 1/10, or 1/5. A serum sample from an atopic dog with *Malassezia* dermatitis and a normal dog were included in each dilution group. Two different dilutions of anti-dog IgE conjugated with HRP were used: 1/500 (tray 1) and 1/100 (tray 2).

All strips showed general background with the strips in tray 2 exhibiting darker staining due to the low dilution of anti-dog IgE. Despite this, no visible bands were present in any of the strips, suggesting an increase in sensitivity was required to obtain adequate antigen signals.

4.4 Protocol 2—introduction of an amplification step to protocol 1

Aims

Due to the lack of sensitivity seen in the previous experiments using a goat anti-dog IgE and chromogenic substrate to detect *Malassezia*-specific IgE, a secondary antibody was introduced in protocol 2 as an amplification step to increase the sensitivity.

Methods

Four pre-wetted strips with separated *Malassezia* proteins were blocked with 1 % skimmed milk in TBS for 1 hour and rinsed with TTBS for 15 minutes. They were then incubated with serum samples either from an atopic dog with *Malassezia* dermatitis or a normal dog diluted 1/100 in dilution buffer for 1 hour (Figure 4.3). After rinsing with TTBS for 15 minutes, the strips were incubated with goat anti-dog IgE diluted 1/1,000 for a further 1 hour and then washed in TTBS for 15 minutes. Higher dilutions of dog sera and anti-dog IgE were used in this experiment because of the background seen in the previous experiments and because a secondary antibody was being used in the following step. A monoclonal anti-goat IgG conjugated with AP was used at dilutions of 1/5,000 or 1/10,000 and the incubation lasted for 1 hour. The strips were washed again in TTBS and developed with alkaline phosphatase substrate, BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium).

Figure 4.3 Experimental design

Strips	1	2	3	4
Blocking	1 % skimmed milk in TBS			
Serum	AM 1/100	N 1/100	AM 1/100	N 1/100
Anti-IgE	1/1,000			
2 nd Ab	1/5,000		1/10,000	

AM: atopic dog with *Malassezia* dermatitis; N: normal dog.

Results

Although a secondary antibody was used in this experiment to amplify the binding signal, there were still no visible bands present, neither in strips incubated with serum from an atopic dog with *Malassezia* dermatitis nor a normal dog (Figure 4.4). This could stem from high dilutions of the reagents and might be improved by increasing their concentrations. However, the low sensitivity of the chromogenic system and a rise in serum consumption indicated that a more sensitive, chemiluminescent detection system should be considered. Protocols 1 and 2 were therefore abandoned due to lack of sensitivity.

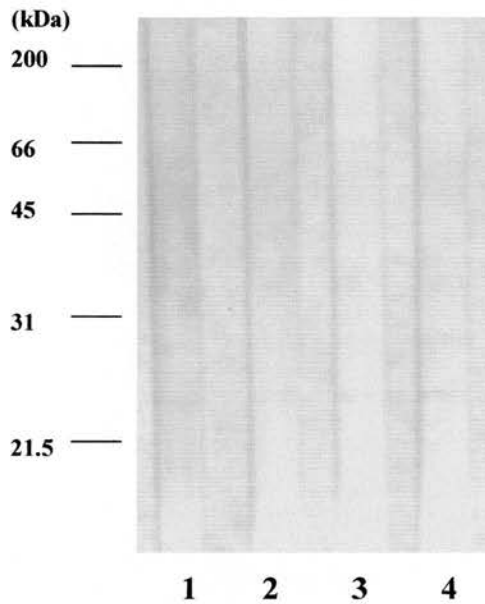


Figure 4.4 Evaluation of using a secondary antibody as an amplification step to enhance antigen signals

Immunoblots showing the effects of adding a secondary antibody, AP conjugated monoclonal anti-goat IgG, on antigen signal amplification. Two different concentrations of anti-goat IgG were evaluated: 1/5,000 (lanes 1 and 2) and 1/10,000 (lanes 3 and 4). Each dilution group comprised a serum sample chosen from an atopic dog with *Malassezia* dermatitis (lanes 1 and 3) and a normal dog (lanes 2 and 4). All strips were incubated with serum diluted 1/100 and then anti-dog IgE diluted 1/1,000.

No improvement of signal enhancement was seen with the dilutions of reagents used in this experiment. The strips showed no staining and this protocol was therefore rejected.

Chemiluminescent methods

4.5 Protocol 3–detection with a monoclonal mouse anti-canine IgE antibody

4.5.1 Assessment of reagent specificity

Aims

The previous experiments demonstrated that chromogenic detection was not sensitive enough to reveal visible bands on the strips. To increase the sensitivity, a chemiluminescent protocol was investigated in this section. Furthermore, the polyclonal goat anti-dog IgE was replaced with a monoclonal mouse anti-dog IgE to ensure maximum specificity of the chemiluminescent method. A multi-channel miniblottedter was also introduced into the methodology because this potentially provides several advantages over cutting a membrane into strips, such as the use of minimal reagent volumes and maximal screening of multiple samples on a single blot.

Methods

An intact membrane containing separated *Malassezia* proteins was pre-wetted with methanol, rinsed with de-ionised water, and blocked with 1 % skimmed milk in TBS for 1 hour. After washing in TTBS for 15 minutes (3 x 5 minute rinses), excess liquid was removed using filter papers with care to avoid excessive drying of the membrane. It was then placed into the miniblottedter with the protein bearing side facing the channels, covered with a foam cushion, and secured by tightening the screws around the miniblottedter as described in the manufacturer's instructions. A water vacuum aspirator was used to remove excess liquid within the channels, and reagents were loaded as soon as possible to prevent the membrane drying out. Approximately 60 µl

of reagents was required to fill a channel. A serum sample from an atopic dog with *Malassezia* dermatitis and a normal dog diluted 1/100 in dilution buffer were then loaded into the channels and allowed to react for 1 hour. A pair of serum samples that had been heat-treated at 56°C for 4 hours to denature IgE was also included for comparison with non-heated samples (Figure 4.5). Any unused channel lying over the membrane was filled with buffer to prevent side leakage and contamination. Dog sera were then aspirated and each channel was flushed with TTBS twice using a vacuum aspirator. The membrane was then incubated with monoclonal mouse anti-dog IgE (D9#3116, kindly donated by Dr. D.J. DeBoer, University of Wisconsin, USA) diluted 1/10,000 for 1 hour, washed with TTBS, and incubated with a HRP conjugated goat anti-mouse IgG antibody diluted 1/1,000 for a further one hour (dilutions determined from preliminary experiments, data not shown). After the channels were flushed with TTBS once, the membrane was removed from the miniblotter and further washed with TTBS for 15 minutes in a tray followed by 2 rinses with TBS. The chemiluminescent substrate for HRP, ECLTM western blotting detection solution (Amersham pharmacia biotech, UK), was then added to cover the whole membrane and allowed to react for 1 minute. Excess reagents were drained off by holding the membrane vertically against a tissue. For visualisation of bands, the membrane was wrapped in cling film, placed in a film cassette with the protein side up and covered with a sheet of autoradiography film (HyperfilmTM ECLTM, Amersham Life Science). The exposure lasted for 2 minutes.

Figure 4.5 Experimental design

Channels	1	2	3	4	5	6	7	8	9	10
Blocking	1 % skimmed milk in TBS									
Serum	AM 1/100	N 1/100	H-AM 1/100	H-N 1/100	Buffer	Buffer	AM 1/100	N 1/100	AM 1/100	N 1/100
Anti-IgE	1/10,000						Buffer	Buffer	1/10,000	
2 nd Ab	1/1,000								Buffer	Buffer

H: heat-treated; AM: atopic dog with *Malassezia* dermatitis; N: normal dog.

Results

Strong signals were present in lanes 1, 3 and 7 where serum from an atopic dog with *Malassezia* dermatitis was used, however, the bands were difficult to interpret due to the diffuse images (Figure 4.6). In contrast, no binding was detected in lanes where normal dog serum was applied (lanes 2, 4 and 8), suggesting that there were some additional proteins in the atopic dog serum that were not present in the normal serum. As compared to the lack of signal in lane 9 where the goat anti-mouse IgG was replaced with buffer, the strong signal shown in lane 7 where anti-dog IgE was omitted indicated that the binding seen in this experiment was mainly generated by the anti-mouse IgG, and not the anti-dog IgE. Since no signal was detected if serum was left out (lanes 5 and 6), the anti-mouse IgG must be binding to serum proteins and not *Malassezia* extracts.

The weaker signal present in lane 3 where heated atopic serum was used compared to non-heated serum (lane 1) suggested that some of the signal in lane 1 was due to binding of anti-dog IgE. The IgE in dog serum would be denatured during the heating process and not recognised by the anti-dog IgE, resulting in a decrease in signal magnitude. However, a stronger binding density was seen in the lane where anti-dog IgE was omitted (lane 7) compared to the other two lanes. One possible

explanation for this phenomenon could be that the binding of anti-mouse IgG was partially blocked by anti-dog IgE.

Taken together, the results of this experiment showed that the goat anti-mouse IgG cross-reacted with serum proteins which were present in higher concentrations in serum from atopic dogs with *Malassezia* dermatitis than from normal dog serum. It is likely that the anti-mouse IgG bound to *Malassezia*-specific IgG in dog serum, as it was demonstrated in chapter 3 that atopic dogs with *Malassezia* dermatitis had a greater IgG response to *M. pachydermatis* than normal dogs.

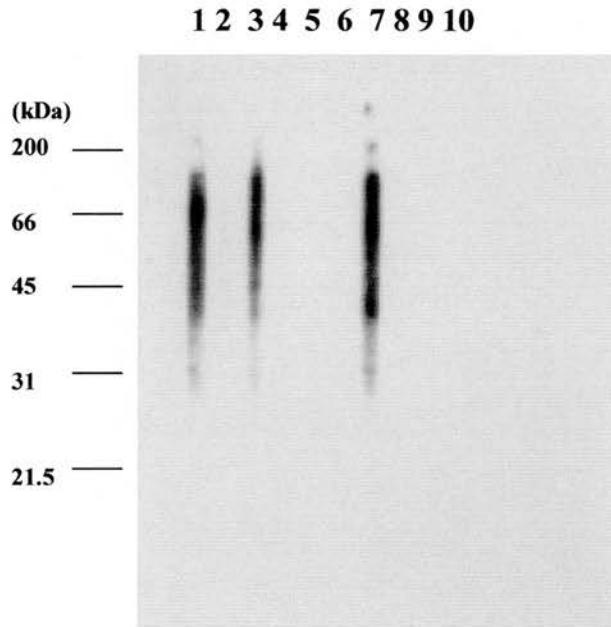


Figure 4.6 Assessment of reagent specificity on a chemiluminescent blot

X-ray film showing the result of reagent specificity evaluated using chemiluminescent detection. A serum sample from an atopic dog with *Malassezia* dermatitis was used in lanes 1, 3, 7 and 9, and a sample from a normal dog was used in lanes 2, 4, 8 and 10. Samples in lanes 3 and 4 were pre-heated at 56°C for 4 hours to denature IgE. In lanes 7 and 8, the anti-dog IgE was replaced with dilution buffer, and in lanes 9 and 10, the conjugated secondary Ab was replaced with dilution buffer.

A high signal was seen in lane 7 where the mouse anti-dog IgE was omitted, indicating that the secondary Ab cross-reacted with other proteins, rather than being specific for mouse IgG, resulting in non-specific signals on the film.

4.5.2 Cross-reactivity of the secondary antibody with canine IgG

Aims

The goat anti-mouse IgG used in the previous experiment was demonstrated to generate non-specific binding on the blots probably due to cross-reactivity between the anti-mouse IgG and canine IgG. The aim of these experiments was to test this hypothesis by directly probing dog sera and purified canine IgG with the goat anti-mouse IgG.

Methods

Since the anti-mouse IgG was specific for the whole mouse IgG molecule, the SDS-PAGE was performed using both non-reducing and reducing conditions with the latter acting as the control. Three different normal dog serum samples (including the one used in the last experiment) and purified dog IgG (Sigma, UK) were diluted 1/100 and 1/20 respectively with PBS and mixed 1 : 1 with non-reducing or reducing sample buffer for separation in a 12 % SDS-PAGE gel. After electrophoresis, one gel was stained with Coomassie blue (section 2.11) and the other was used for electrophoretic transfer and immunoblotting (sections 2.12 and 2.13).

The molecular weight standards transferred onto the membrane were cut off and stained with Coomassie blue. The rest of the membrane was blocked for 1 hour with 1 % skimmed milk in TBS. After washing with TTBS for 15 minutes (3 x 5 minute rinses), the membrane was incubated for 1 hour with the goat anti-mouse IgG diluted 1/1,000 in dilution buffer (1 % skimmed milk in TTBS). The membrane was washed again in TTBS for a further 15 minutes and then developed with DAB peroxidase substrate.

Results

The protein profiles of normal dog serum and purified dog IgG obtained under non-reducing and reducing conditions in an SDS-PAGE gel are shown in Figure 4.7 A-B. Under non-reducing condition, purified dog IgG exhibited a strong single band at a molecular weight of slightly over 200 kDa and this band was present in all three serum samples, although there was a difference in strength between the samples. (Figure 4.7 A). In contrast, two separated bands were shown in the gel when the samples were mixed with reducing buffer (Figure 4.7 B), indicating that the IgG molecule was cleaved into heavy and light chains.

Clear bands were shown in every sample on the non-reducing blot, which corresponded to the high molecular weight band shown in the gel (Figure 4.7 C). However, no binding was detected on the reducing blot (Figure 4.7 D). This confirmed that the goat anti-mouse IgG was specific for the whole IgG molecule, but did not detect the heavy or light chains specifically. Nevertheless, the binding on the non-reducing blot verified that the anti-mouse IgG cross-reacted with canine IgG and was able to bind to IgG in dog sera. Thus, protocol 3 was abandoned due to lack of specificity.

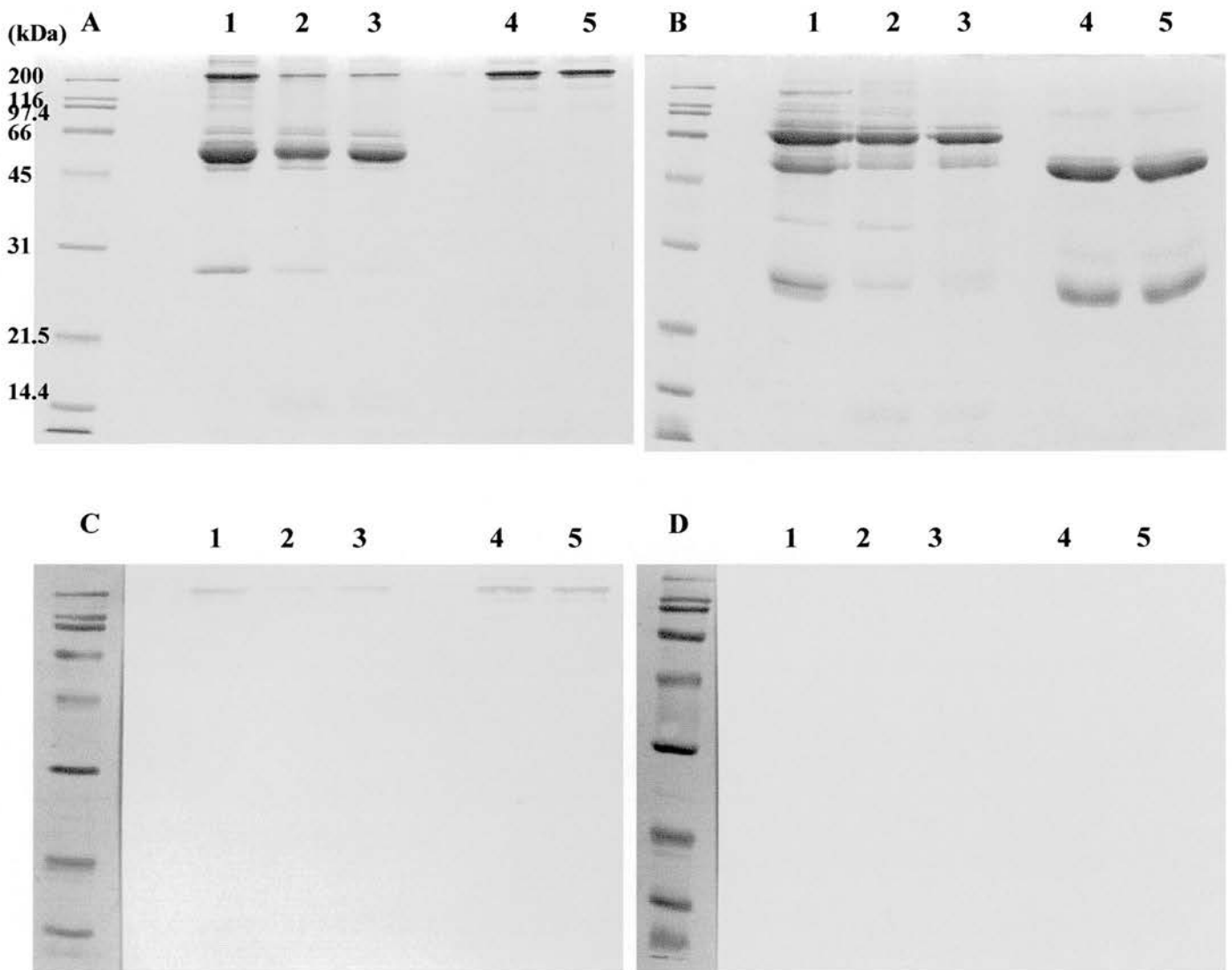


Figure 4.7 A-D Demonstration of non-specific binding between the HRP conjugated goat anti-mouse IgG and purified canine IgG

A: Three different serum samples from normal dogs (lanes 1-3) and purified canine IgG loaded in duplicate (lanes 4 and 5) were analysed using non-reducing sample buffer in an SDS-PAGE gel. The whole IgG molecule exhibited a strong single band at the top of the gel and this was present in each sample. B: An SDS-PAGE gel showing the same samples analysed using reducing sample buffer. Two clear bands in lanes 4 and 5 represented heavy and light chains of canine IgG.

C: Cross-reactivity between the goat anti-mouse IgG and canine IgG demonstrated by immunoblotting. Binding was shown in each lane, confirming that the anti-mouse IgG cross-reacted with canine IgG and bound to IgG in dog sera. D: Immunoblot showing the anti-mouse IgG did not react to either heavy or light chain of canine IgG.

4.6 Protocol 4—evaluation of a monoclonal bovine anti-mouse IgG as the secondary antibody

4.6.1 Binding of bovine anti-mouse IgG1 to mouse anti-canine IgE

Aims

Due to the cross-reactivity between the goat anti-mouse IgG and canine IgG as demonstrated in the previous sections, a monoclonal bovine anti-mouse IgG1 antibody (Serotec, Oxford, UK) was evaluated as the secondary antibody for immunoblotting. The aim of this experiment was to determine if the antibody could bind to the primary antibody, monoclonal mouse anti-dog IgE, using dot blotting.

Methods

A piece of dry membrane was cut into a suitable size and the sites of application of the antibody solutions were marked with a pencil. The use of a dry membrane facilitated the confinement of antibody solutions to a small area and prevented diffusion. A volume of 0.5 μ l of monoclonal mouse anti-dog IgE (D9#3116, kindly donated by Dr. D.J. DeBoer, University of Wisconsin, USA) diluted 1/1,000, 1/5,000 and 1/10,000 in dilution buffer was dropped at the marked areas and allowed to dry. After the solutions had dried, another 0.5 μ l of various dilutions of anti-dog IgE was dropped at the same places and this was repeated 10 times. Mouse IgG1 κ (Sigma, UK) at a dilution of 1/1,000 was used as the positive control, and dilution buffer for the negative control. After the last drops were air-dried, the membrane was blocked with 1 % skimmed milk in TBS for 1 hour and washed with TTBS for 15 minutes (3 x 5 minute rinses). It was then cut into four strips and incubated with HRP conjugated bovine anti-mouse IgG1 at dilutions of 1/1,000, 1/5,000 and 1/10,000 (Figure 4.8). After a 1-hour incubation, the strips were washed again in TTBS and

then developed with a chemiluminescent substrate, ECL™, as described in section 4.5.1. The exposure lasted for 5 minutes.

Figure 4.8 Experimental design

Mouse anti-dog IgE	Bovine anti-mouse IgG1			
	1/1,000	1/5,000	1/10,000	1/1,000
1/1,000				Mouse IgG1κ 1/1,000
1/5,000				Buffer
1/10,000				

Results

The binding of bovine anti-mouse IgG1 to mouse anti-dog IgE is shown in Figure 4.9. A clear signal was seen at the location of the mouse IgG1κ, but no binding was detected at the site of the dilution buffer. This indicated that the binding exhibited in this experiment was specific. Strong intensity of binding was present when anti-dog IgE was used at a dilution of 1/1,000 regardless of the dilution of anti-mouse IgG1. Higher dilutions of both antibodies resulted in a reduction of signal. The results of this experiment confirmed that bovine anti-mouse IgG1 was capable of binding to mouse anti-dog IgE.

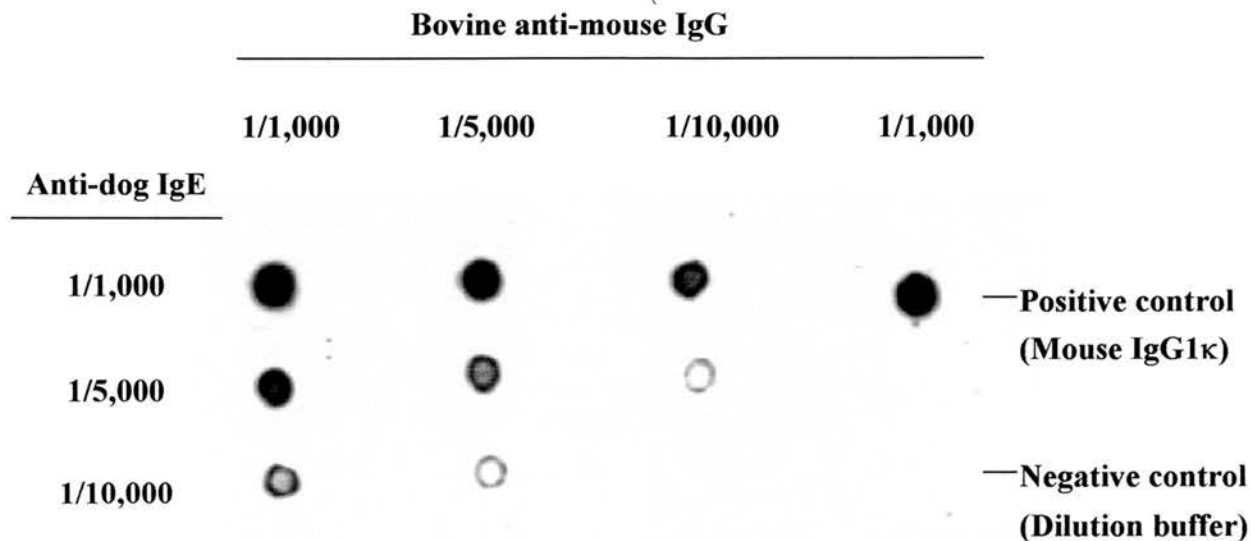


Figure 4.9 Demonstration of binding between bovine anti-mouse IgG1 and mouse anti-dog IgE by dot blotting

Three different dilutions of bovine anti-mouse IgG1 and mouse anti-dog IgE were used to test their binding activity. Strong signals were present at the positions where mouse IgG1κ and lower dilutions of both antibodies were used. In contrast, no binding was shown at the site of the dilution buffer, indicating that the signals exhibited in this experiment were specific. These results showed that bovine anti-mouse IgG1 was able to bind to mouse anti-dog IgE.

4.6.2 Investigation into cross-reactivity of bovine anti-mouse IgG1 with canine IgG

Aims

The aim of this experiment was to examine the specificity of the bovine anti-mouse IgG1 antibody to further evaluate the feasibility of using this antibody in *Malassezia* blots.

Methods

Mouse IgG1 κ (Sigma, UK), a normal dog serum sample, and purified dog IgG (Sigma, UK) were diluted 1/10, 1/100, and 1/20 respectively with PBS and mixed 1 : 1 with non-reducing sample buffer. They were then separated in a 12 % SDS-PAGE gel and blotted onto a membrane. The membrane was pre-wetted with methanol, rinsed with de-ionised water and blocked with 1 % skimmed milk in TBS for 1 hour. After washing with TTBS for 15 minutes (3 x 5 minute rinses), the membrane was incubated with bovine anti-mouse IgG1 diluted either 1/1,000 or 1/10,000 for 1 hour. It was washed again in TTBS for a further 15 minutes, and then developed with DAB peroxidase substrate or ECLTM chemiluminescent substrate.

Results

The anti-mouse IgG1 was shown to bind to mouse IgG1 κ , but not to either normal dog serum or purified dog IgG on a DAB developed blot when used at a dilution of 1/1,000 (Figure 4.10 A). However, cross-reactivity with canine IgG was shown on a chemiluminescent blot when the same dilution of anti-mouse IgG1 was used (Figure 4.10 B). This confirmed that chemiluminescent reagents were more sensitive than chromogenic reagents, and were able to detect weaker signals. Nevertheless, when

used at the dilution of 1/10,000, the binding of anti-mouse IgG1 to normal dog serum and purified dog IgG was not present on the chemiluminescent blot under the same exposure conditions (Figure 4.10 C). This suggested that the cross-reactivity might be overcome by using this antibody at a higher dilution.

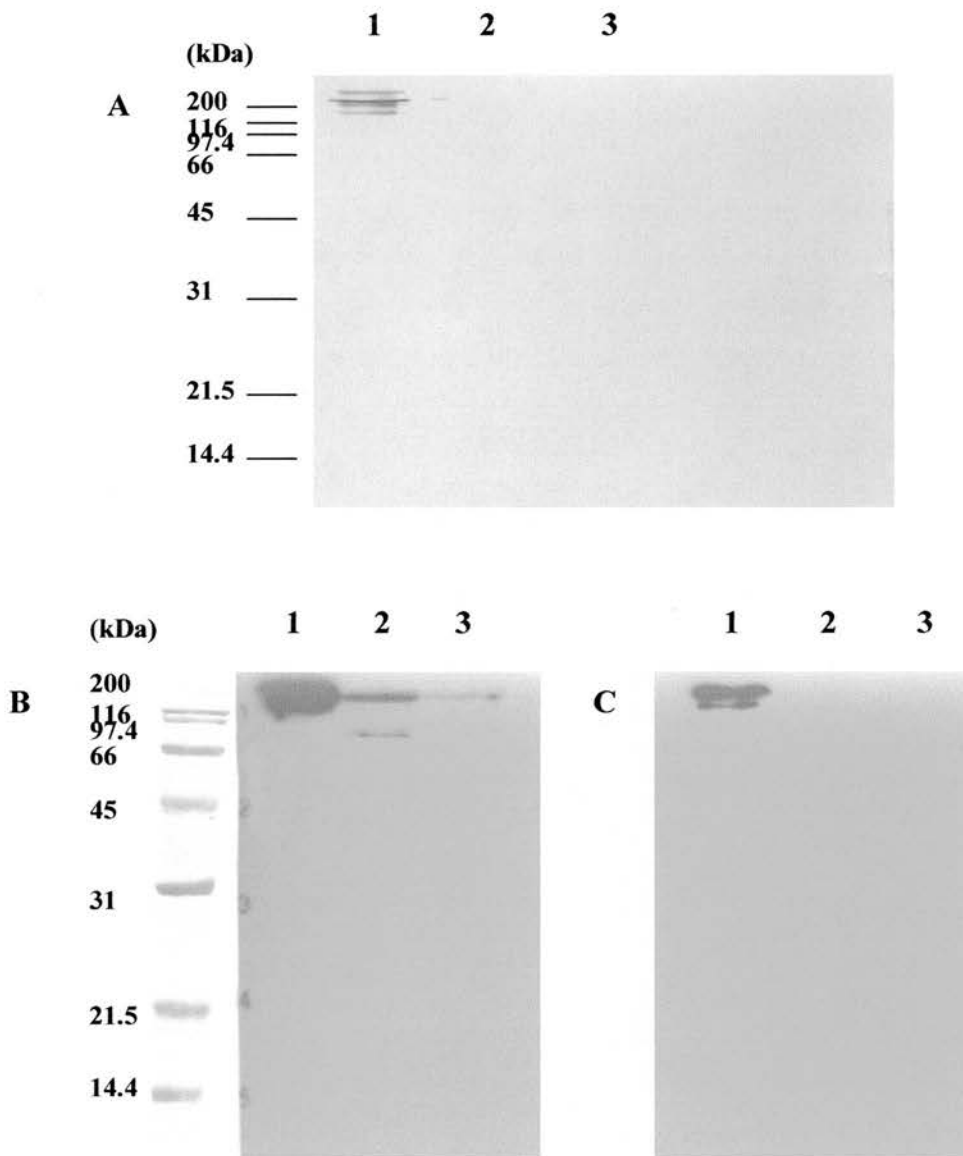


Figure 4.10 A-C Evaluation of the specificity of bovine anti-mouse IgG1 using chromogenic and chemiluminescent detection

A: Immunoblot incubated with bovine anti-mouse IgG1 diluted 1/1,000 and developed with DAB peroxidase substrate. Lane 1: mouse IgG1 κ ; lane 2: normal dog serum; lane 3: purified dog IgG. The binding in lane 1 verified that the anti-mouse IgG1 was specific for mouse IgG1. No cross-reactivity with canine IgG was seen in lanes 2 and 3 using chromogenic detection. B: Immunoblot incubated with bovine anti-mouse IgG1 diluted 1/1,000 and developed with chemiluminescent substrate. Cross-reactivity of anti-mouse IgG1 to canine IgG was seen in lanes 2 and 3 using the more sensitive detection method. C: Immunoblot incubated with bovine anti-mouse IgG1 diluted 1/10,000 and developed with chemiluminescent substrate. The cross-reactivity was not detectable (lanes 2 and 3) on the chemiluminescent blot when the anti-mouse IgG1 was used at a higher dilution.

4.6.3 Optimisation of anti-mouse IgG1 dilution to overcome cross-reactivity

Aims

The previous experiments demonstrated that the bovine anti-mouse IgG1 cross-reacted with canine IgG when a lower dilution of the antibody was used with a chemiluminescent substrate. However, the cross-reactivity was not detectable when anti-mouse IgG1 was used at a higher dilution. Hence, this experiment was designed to evaluate the possibility of overcoming the cross-reactivity without loss of sensitivity by optimising the dilution of anti-mouse IgG1 for *Malassezia* immunoblotting.

Methods

An intact membrane containing *Malassezia* extract was pre-wetted and blocked with 1 % skimmed milk for 1 hour. After washing in TTBS for 15 minutes (3 x 5 minute rinses), the membrane was placed into the multi-channel miniblitter as described in section 4.5.1. To conserve the supply of serum from atopic dogs with *Malassezia* dermatitis, a serum sample from a normal dog that had shown a strong signal in a preliminary experiment (data not shown) was used for this experiment. The serum was diluted 1/5 in dilution buffer and loaded into channels. An aliquot of the same sample that had been heated at 56°C for 4 hours was also included. Each incubation step lasted for 1 hour. After flushing with TTBS, the majority of the channels were filled with mouse anti-dog IgE diluted 1/10,000 and the others dilution buffer (Figure 4.11). The channels were further washed with TTBS and filled with bovine anti-mouse IgG1 diluted 1/1,000, 1/2,000, 1/4,000, 1/6,000, 1/8,000 and 1/10,000. At the end of a 1-hour incubation, excess reagents were aspirated followed by another flush with TTBS. The membrane was then removed from the miniblitter, and further

washed in TTBS for 15 minutes. After rinsing with TBS, it was developed with chemiluminescent substrate as described in section 4.5.1 and the exposure lasted for 5 minutes.

Figure 4.11 Experimental design

Channels	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Blocking	1 % skimmed milk in TBS																	
Serum 1/5	N	H	N	N	H	N	N	H	N	N	H	N	N	H	N	N	H	N
Anti-IgE	1/10,000		B	1/10,000		B	1/10,000		B	1/10,000		B	1/10,000		B	1/10,000		B
2 nd Ab	1/1,000		1/2,000			1/4,000			1/6,000			1/8,000			1/10,000			

N: normal dog; H: heat-treated; B: dilution buffer.

Results

The effect of anti-mouse IgG1 concentrations on its sensitivity and specificity in *Malassezia* blots is shown in Figure 4.12. The intensity of signal decreased gradually as the dilution of anti-mouse IgG1 increased. However, although using more diluted antibody solution helped to eliminate non-specific binding, it also reduced the total signal level. There was no noticeable difference between channels where mouse anti-dog IgE or dilution buffer was used at any dilution of conjugate. The intensity of the signal shown by non-heated and heated serum samples was also very similar. These results indicated that the binding shown on the blot was generated predominantly by the non-specific binding of anti-mouse IgG1 to canine IgG in dog serum. In summary, this experiment did not demonstrate a dilution of anti-mouse IgG1 that could be used in a *Malassezia* blot to generate clear, but specific signals. This protocol was therefore abandoned due to lack of specificity.

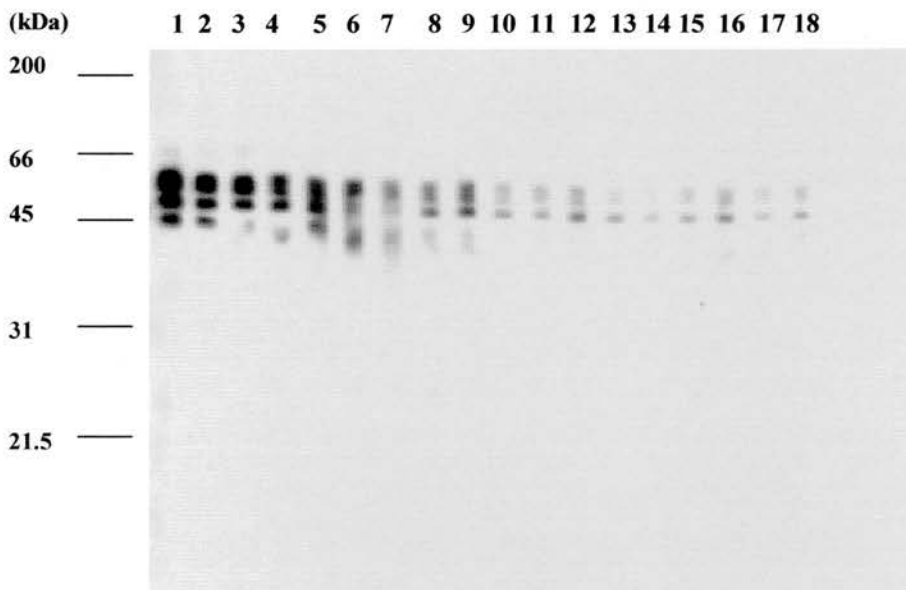


Figure 4.12 The effect of bovine anti-mouse IgG1 concentrations on its sensitivity and specificity in *Malassezia* blots

X-ray film showing that the cross-reactivity of anti-mouse IgG1 to dog serum could not be eliminated by dilution. Lanes 2, 5, 8, 11, 14 and 17: heat-treated normal dog serum. The others: non-heated serum. Lanes 3, 6, 9, 12, 15 and 18: mouse anti-dog IgE was replaced with dilution buffer. Every dilution group of bovine anti-mouse IgG1 comprised three lanes, and the groups from left to right were 1/1,000 (lanes 1-3), 1/2,000 (lanes 4-6), 1/4,000 (lanes 7-9), 1/6,000 (lanes 10-12), 1/8,000 (lanes 13-15) and 1/10,000 (lanes 16-18). No substantial difference was seen between the three lanes in each dilution group, and they all showed decreased signal when more diluted conjugate was used. No dilution tested in this experiment generated specific binding.

4.7 Protocol 5–evaluation of a biotinylated recombinant human IgE receptor alpha chain as a detection reagent

4.7.1 Detection with avidin reagent 1- Vectastain® Elite ABC kit

4.7.1.1 *Binding of human IgE receptor to canine IgE*

Aims

In this section, a biotinylated recombinant human IgE receptor alpha chain was evaluated for its suitability as a detection reagent. In theory, the IgE receptor should allow total specificity for IgE, eliminating the problems of cross-reactivity with canine IgG as seen in the previous experiments. Owing to the human origin of the IgE receptor, it was important to demonstrate that this receptor was capable of binding to canine IgE in the immunoblotting system used in this study.

Methods

Purified dog IgE (Bethyl Laboratories, Montgomery TX, USA) was diluted ten-fold with PBS and mixed 1: 1 with non-reducing sample buffer. It was then separated in an SDS-PAGE gel and transferred onto a membrane. The membrane was blocked with 1 % skimmed milk in TBS for 1 hour, and washed in TTBS for 15 minutes (3 x 5 minute rinses). It was then probed with the human IgE receptor diluted 1/1,000 in dilution buffer for 1 hour followed by another 15-minute wash. Bound receptor was detected by incubating the membrane for 1 hour with Vectastain® Elite ABC reagents (Vector Laboratories, Inc., Burlingame, CA, USA) containing Avidin DH and biotinylated horseradish peroxidase H. After further washing with TTBS for 15 minutes, the membrane was rinsed with TBS and developed with ECL™ chemiluminescent substrate. The exposure lasted for 10 minutes.

Results

Binding of the human IgE receptor to purified dog IgE is shown in two lanes where dog IgE was used at the dilutions of 1/10 and 1/100 with the former exhibiting stronger binding intensity (Figure 4.13). This indicated that the biotinylated human IgE receptor was able to bind to dog IgE and that binding was detectable using the avidin and chemiluminescent reagents. The receptor was therefore used in the following immunoblotting experiments.

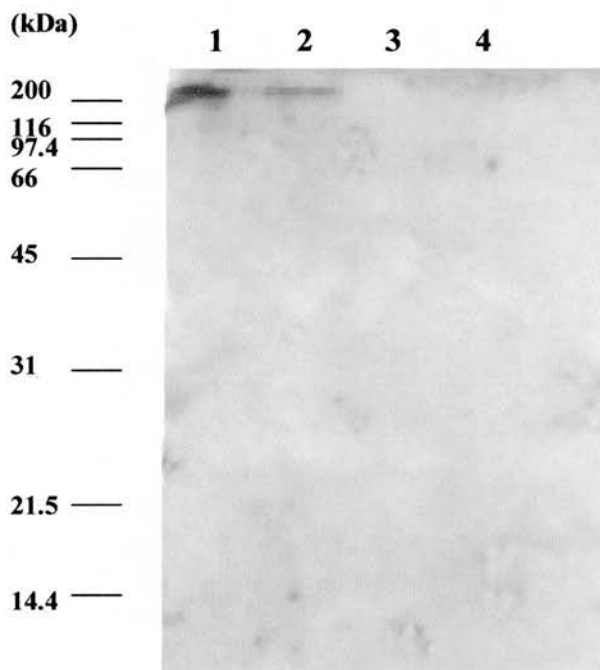


Figure 4.13 Demonstration of binding between biotinylated recombinant human IgE receptor alpha chain and purified canine IgE

Chemiluminescent blot showing the binding of human IgE receptor reagent to dog IgE. Lanes 1-4: purified dog IgE diluted 1/10, 1/100, 1/1,000, and 1/10,000, respectively. One high molecular weight band was present in lanes 1 and 2, indicating that the IgE receptor reagent was able to bind to dog IgE.

4.7.1.2 *Assessment of reagent specificity in Malassezia blots*

Aims

Having demonstrated the potential use of the human IgE receptor reagent in immunoblotting, various combinations of reagents were tested in *Malassezia* blots to assess their specificity. Also, in order to prevent the uncertainty of reagent side leaking caused by using the miniblotted, and to ensure that every control was well separated, the use of a miniblotted was abandoned and strips containing separated *Malassezia* proteins were used in subsequent experiments.

Methods

Eight strips containing separated *Malassezia* proteins were pre-wetted and blocked with 5 % skimmed milk in TBS for 1 hour. Due to the dark background shown on the blots in preliminary experiments (data not shown), a higher concentration of skimmed milk was used to block the strips. After washing with TTBS for 15 minutes, two strips were incubated for 1 hour with a serum sample from an atopic dog with *Malassezia* dermatitis diluted 1/10 in dilution buffer, and a serum sample from a normal dog at the same dilution was used for another two strips. The others were incubated with dilution buffer alone (Figure 4.14). The strips were washed again with TTBS for 15 minutes. Half of them were probed with the IgE receptor reagent diluted 1/500 for 1 hour followed by washing in TTBS for a further 15 minutes. Vectastain® Elite ABC reagents were then applied to the majority of the strips and allowed to react for 30 minutes. After another 15-minute wash, the strips were rinsed with TBS and developed with ECL™ chemiluminescent substrate. The exposure lasted for 1 minute.

Figure 4.14 Experimental design

Strips	1	2	3	4	5	6	7	8
Blocking	5 % skimmed milk in TBS							
Serum	Buffer	AM 1/10	N 1/10	Buffer	Buffer	AM 1/10	N 1/10	Buffer
FcεRIα	Buffer	Buffer	Buffer	1/500				Buffer
ABC	Applied							Buffer

AM: atopic dog with *Malassezia* dermatitis; N: normal dog.

Results

Strong binding in the high molecular weight range was shown in every strip except the last strip which was incubated with buffer alone in all three blotting steps (Figure 4.15). The absence of binding in the last strip indicated that the chemiluminescent substrate did not bind to proteins on the strips. However, the binding exhibited on the strip incubated with ABC reagents only (lane 1) indicated that the ABC reagents bound to components in the *Malassezia* extract non-specifically. As the binding pattern was consistent in all strips, it was inferred that all the binding shown in this experiment was generated mainly from the cross-reactivity of ABC reagents with *Malassezia* proteins. However, more specific binding activity might have been overshadowed by this strong cross-reaction. The most likely explanation was that the *Malassezia* extract used in this study, produced by grinding whole organisms with glass beads, could contain endogenous biotin, protein-bound biotin or other avidin binding proteins that reacted with the ABC reagents. In order to overcome this cross-reactivity, various reagents were used to block it in the following experiment.

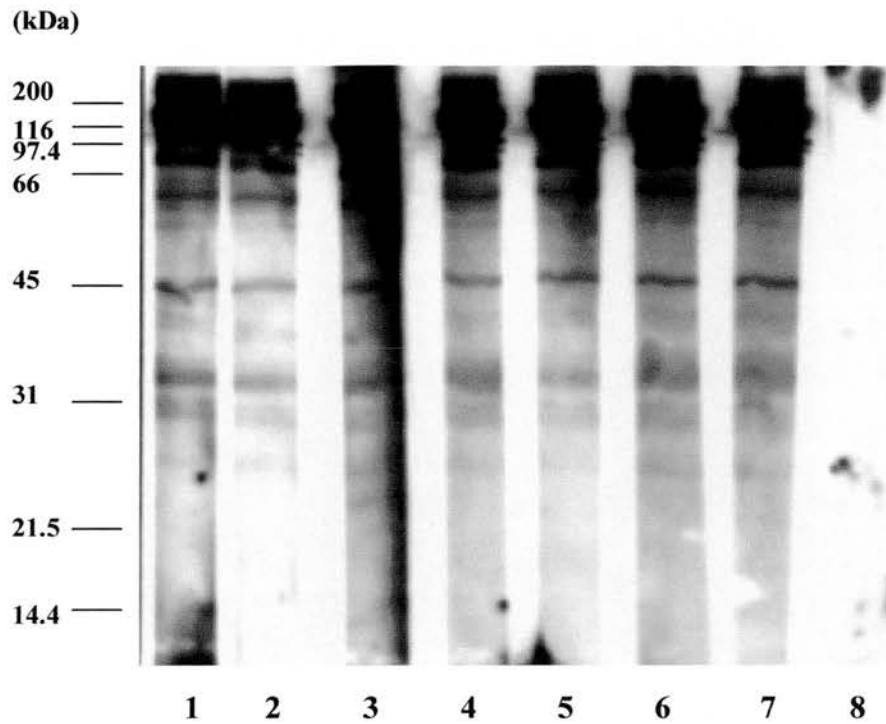


Figure 4.15 Assessment of the specificity of the IgE receptor and ABC reagents in *Malassezia* blots

Chemiluminescent immunoblots showing the results of strips probed with various combinations of reagents. Lanes 2 and 6: serum from an atopic dog with *Malassezia* dermatitis. Lanes 3 and 7: serum from a normal dog. IgE receptor was used in lanes 4-7, and ABC reagents were applied to all strips except lane 8. No binding was detected in lane 8, indicating that the chemiluminescent substrate did not bind to proteins on the strips. The first strip was not probed with either dog serum or IgE receptor, yet incubation with ABC reagents resulted in very strong binding signals, suggesting cross-reactivity between the ABC reagents and certain components in the *Malassezia* extract, possibly endogenous protein-bound biotin.

4.7.1.3 Evaluation of various blocking reagents

Aims

Cross-reactivity between the avidin reagent, Vectastain® Elite ABC, and the *Malassezia* extract resulted in very strong signals on chemiluminescent blots as shown in the previous experiment. A variety of blocking reagents were therefore evaluated to try and block avidin binding sites in the *Malassezia* extract, and to reduce the background on the strips. These included skimmed milk, Tween 20, Tween 80, casein solution, and an avidin/biotin blocking kit.

Methods

Strips containing separated *Malassezia* proteins were pre-wetted and blocked with various reagents for 1 hour (Figure 4.16). Both higher concentrations of skimmed milk and Tween 20 were used in this experiment. The skimmed milk powder was used at a concentration of 10 % and Tween 20, 0.1%. TBS containing Tween 20 or Tween 80 (TTBS) was used to dissolve the milk powder as it has been reported that TTBS generally produces a clean background (Gallagher *et al.* 1998). The blocking solutions used were 10 % skimmed milk in TBS containing 0.1 % Tween 20 (TTBS 1), TTBS 1 alone, 10 % skimmed milk in TBS containing 0.5 % Tween 80 (TTBS 2), TTBS 2 alone, and 0.5 % casein solution containing 0.1 % Tween 20 (Vector Laboratories, Inc., Burlingame, CA, USA). The strips were washed for 15 minutes with TTBS1 or TTBS 2 depending on which reagent was used for blocking, and one strip from each blocking group was incubated with avidin D solution from the avidin/biotin blocking kit (Vector Laboratories, USA) for 10 minutes to allow the avidin to react with biotin on the strips. The others were incubated with buffer alone. After washing with TTBS for another 15 minutes, the strips blocked with avidin D

solution were incubated with biotin blocking solution from the same kit for a further 10 minutes to block the remaining biotin binding sites on the avidin. The strips were washed again in TTBS for 15 minutes and incubated with ABC reagents for 30 minutes. A strip was incubated with buffer without ABC reagents to act as a control. They were further washed in TTBS for 15 minutes, rinsed with TBS, and developed with ECL™ chemiluminescent substrate. The exposure lasted for 1 minute.

Figure 4.16 Experimental design

Strips	1	2	3	4	5	6	7	8	9	10	11
Blocking	10 % skimmed milk in TTBS1		TTBS1	TTBS1	10 % skimmed milk in TTBS2		TTBS2	TTBS2	casein	casein	TTBS1
A/B kit	Applied	Buffer	Applied	Buffer	Applied	Buffer	Applied	Buffer	Applied	Buffer	Buffer
ABC	Applied										Buffer

A/B kit: avidin/biotin blocking kit; TTBS1: TBS containing 0.1 % Tween 20; TTBS 2: TBS containing 0.5 % Tween 80.

Results

The comparison of the various blocking reagents is shown in Figure 4.17. Strong signals were present in all strips except the negative control that was not incubated with ABC reagents. No substantial difference was shown between any of the strips, indicating that none of the blocking reagents blocked the avidin binding sites in the *Malassezia* extract effectively. There were two possible reasons for this. First, the avidin binding sites in the extract might have outnumbered the avidin in the blocking reagents. Secondly, the ABC reagents were claimed to be very sensitive which might have magnified the cross-reactivity signals. Due to the difficulties in optimising the use of reagents in the ABC kit, an alternative avidin reagent was investigated.

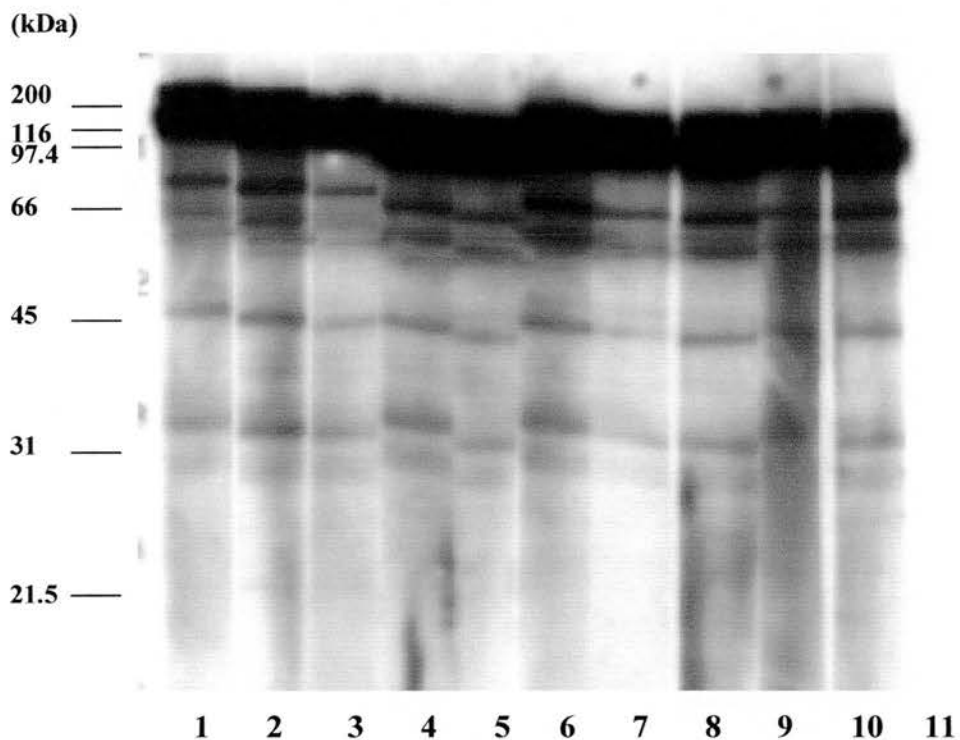


Figure 4.17 Evaluation of various blocking reagents to block the cross-reactivity between ABC reagents and *Malassezia* extract

Lanes 1 and 2 were blocked with 10 % skimmed milk in TBS containing 0.1 % Tween 20 (TTBS1). Lanes 3, 4, and 11: TTBS1 alone. Lanes 5 and 6: 10 % skimmed milk in TBS containing 0.5 % Tween 80 (TTBS2). Lanes 7 and 8: TTBS2 alone. Lanes 9 and 10: casein solution. The strips in lanes 1, 3, 5, 7, 9 were further blocked with avidin/biotin blocking reagents. The ABC reagents were applied to lanes 1-10. None of the blocking reagents tested in this experiment successfully blocked the cross-reactivity of ABC reagents with the *Malassezia* extract.

4.7.2 Detection with avidin reagent 2- Extravidin®

4.7.2.1 Assessment of reagent specificity

Aims

Given the failure of various blocking reagents to block cross-reactivity of ABC reagents with the *Malassezia* extract as demonstrated in the previous experiment, and the limited possibility to adjust the ABC reagent concentrations, the feasibility of using another avidin reagent, Extravidin® (Sigma, UK), in *Malassezia* blots was evaluated. Extravidin® is a modified form of affinity purified avidin and it combines the advantages of high specific activity of avidin with the low background staining of streptavidin. The aim of this experiment was to determine if Extravidin® cross-reacted with *Malassezia* proteins.

Methods

Five pre-wetted strips with separated *Malassezia* proteins were blocked with 5 % skimmed milk in TBS containing 0.1 % Tween 20 (TTBS) for 1 hour and washed in TTBS for 15 minutes. Three of the strips were incubated with a serum sample from an atopic dog with *Malassezia* dermatitis diluted 1/10 in dilution buffer (1 % skimmed milk in TTBS) and the other two strips with buffer alone for 1 hour (Figure 4.18). After a further wash in TTBS for 15 minutes, the IgE receptor diluted 1/500 was applied to three strips, and allowed to react for 1 hour. The others were incubated in buffer. The strips were washed again in TTBS for 15 minutes, and the majority were then incubated with Extravidin® diluted 1/1,000 for a further hour, followed by another 15-minute wash. The strip incubated in buffer without Extravidin® was used as the negative control. The chemiluminescent detection was performed as described in section 4.5.1, and the exposure lasted for 2 minutes.

Figure 4.18 Experimental design

Strips	1	2	3	4	5
Blocking	5 % skimmed milk in TTBS (0.1 % Tween 20)				
Serum	AM 1/10	AM 1/10	AM 1/10	Buffer	Buffer
FcεRIα	1/500	1/500	Buffer	1/500	Buffer
Extravidin®	1/1,000	Buffer	1/1,000	1/1,000	1/1,000

AM: atopic dog with *Malassezia* dermatitis.

Results

All the strips incubated with Extravidin® exhibited very strong and consistent binding to high molecular weight bands with a few bands in the lower molecular weight range between 31 and 66 kDa (Figure 4.19). In contrast to the absence of binding on the strip incubated in buffer without Extravidin®, the binding shown on the strip incubated with only Extravidin® indicated that this reagent also cross-reacted with components in the *Malassezia* extract. The very similar results generated by two different reagents suggested that the sources of cross-reactivity could be the same, and it further increased the possibility that it was endogenous protein bound biotin in the extract. Despite the cross-reactivity of Extravidin® with the *Malassezia* extract, it was possible that the problem could be overcome by increasing the dilution of the reagent and optimising other factors in the blotting procedure.

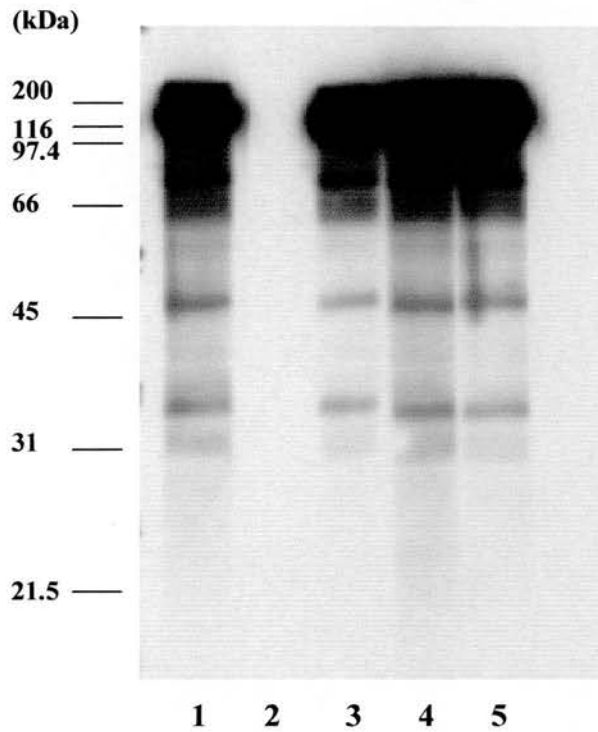


Figure 4.19 Assessment of the specificity of the IgE receptor and Extravidin® in *Malassezia* blots

Immunoblots showing the results of strips probed with various combinations of reagents. The strips in lanes 1-3 were incubated with serum from an atopic dog with *Malassezia* dermatitis. The strips in lanes 1, 2, and 4 were then incubated with IgE receptor. Extravidin® was applied to all strips except lane 2. The strips that were not probed with working reagents were incubated with buffer alone. The binding on the strip incubated without serum and IgE receptor, but with Extravidin® (lane 5) indicated that Extravidin® cross-reacted with the *Malassezia* extract.

4.7.2.2 Evaluation of optimising various factors to overcome cross-reactivity of Extravidin® with the Malassezia extract

Aims

The signal intensity of cross-reactivity between Extravidin® and *Malassezia* proteins depends on the concentration of *Malassezia* extract, the effectiveness of blocking, the working concentration of Extravidin®, and the length of exposure. In order to overcome the cross-reactivity, various adjustments were to be made to these factors to determine the optimal conditions.

Methods

Due to the strong non-specific binding shown by both ABC reagents and Extravidin (sections 4.7.1.2. and 4.7.2.1), and the failure of an avidin/biotin blocking kit to block it (see section 4.7.1.3), the protein concentration of the *Malassezia* extract used in an SDS-PAGE gel was reduced by half. Briefly, *Malassezia* extract prepared with glass beads (60 µg, 150 µl of 1/40 dilution) was mixed with reducing sample buffer and a final volume of 300 µl was added into a broad well running across the top of the gel.

Four strips containing separated *Malassezia* proteins were pre-wetted and blocked with 5 % skimmed milk in TBS containing 0.1 % Tween 20 (TTBS) for 1 hour, followed by washing with TTBS for 15 minutes. All the strips were further blocked with reagents in the avidin/biotin blocking kit, consisting of 10-minute incubations of avidin D solution followed by biotin solution for another 10 minutes with a 15 minute wash in TTBS after each incubation step. Unlike the blocking reagents used in section 4.7.1.3, in which both avidin D and biotin solutions were prepared by

adding one drop of the stock solution into 5 ml of TTBS as suggested in the manufacturer's instructions, 10 drops of each solution was used in the same volume of TTBS in this experiment. This was intended to block all the avidin binding sites in the *Malassezia* extract by providing an excess of avidin in the blocking solution. Each strip was then incubated for 1 hour with a different dilution of Extravidin® in dilution buffer (1 % skimmed milk in TTBS), including 1/1,000, 1/2,000, 1/5,000, and 1/10,000. After further washes with TTBS for 15 minutes, the strips were rinsed with TBS and developed with ECL™ chemiluminescent substrate. The exposure lasted for 2 minutes.

Figure 4.20 Experimental design

Strips	1	2	3	4
Blocking	5 % skimmed milk in TTBS (0.1 % Tween 20)			
A/B kit	Applied (10 drops each in 5 ml TTBS)			
Extravidin®	1/1,000	1/2,000	1/5,000	1/10,000

A/B kit: avidin/biotin blocking kit.

Results

By reducing the amount of *Malassezia* extract in the gel, increasing the concentration of blocking reagents, and increasing the dilution of Extravidin®, a successful blocking of cross-reactivity between Extravidin® and the *Malassezia* extract was achieved in three of the strips (Figure 4.21). No binding was detected on strips incubated with Extravidin® diluted 1/2,000-1/10,000. Although at the dilution of 1/1,000 there was still strong binding present in the high molecular weight range, the strip showed clear background and the bands with lower molecular weights seen in the previous experiments were not present. These results confirmed that the component in the *Malassezia* extract to which the two avidin reagents reacted was

endogenous biotin. A *Malassezia* blot prepared using 60 µg of extract, 0.1 % Tween 20, 10 x avidin/biotin blocking solutions, and an Extravidin® dilution of 1/2,000 was therefore used in subsequent experiments.

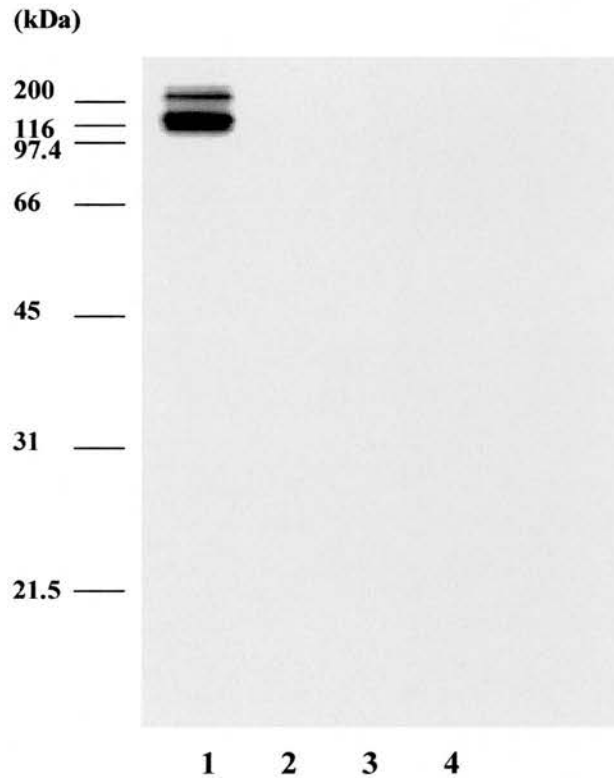


Figure 4.21 Optimisation of Extravidin[®] dilution to block its cross-reactivity with the *Malassezia* extract

The strips in lanes 1-4 were incubated with Extravidin[®] diluted 1/1,000, 1/2,000, 1/5,000, and 1/10,000, respectively. A strong signal was present in the high molecular weight range in lane 1. However, no binding was detected in lanes 2-4, indicating that the cross-reactivity between Extravidin[®] and *Malassezia* extract could be blocked by using the avidin reagent at higher dilutions.

4.7.2.3 Using the FcεRIα reagent and Extravidin® in Malassezia immunoblots

Aims

It had been demonstrated in the previous experiment that the cross-reactivity between Extravidin® and *Malassezia* extract could be overcome by optimising various reagents. The aim of this experiment was to further examine whether cross-reactivity would be a problem in *Malassezia* blots using dog sera and detection reagents.

Methods

A membrane comprising separated *Malassezia* proteins was prepared using 60 µg of the extract in an SDS-PAGE gel and blotted onto the membrane. The membrane was cut into strips and each strip was blocked for 1 hour with 5 % skimmed milk in TBS containing 0.1 % Tween 20 (TTBS), followed by washing in TTBS for 15 minutes. They were further blocked with 10x avidin/biotin blocking reagents for 10 minutes each with a 15 minute wash after each blocking step. Three of the strips were incubated with a serum sample from an atopic dog with *Malassezia* dermatitis diluted 1/10 in dilution buffer (1 % skimmed milk in TTBS) and the other two strips with buffer alone for 1 hour (Figure 4.22). The strips were washed again in TTBS for 15 minutes, and three of the strips were incubated with the IgE receptor diluted 1/50 for 1 hour. The others were incubated in buffer. A lower dilution of IgE receptor reagent was used in this experiment in order to increase the sensitivity after excessive blocking. After a further wash in TTBS for 15 minutes, the majority of the strips were then incubated with Extravidin® diluted 1/2,000 for a further hour, followed by another 15-minute wash. The strip incubated in buffer without Extravidin® was used as the negative control. All strips were then rinsed with TBS and developed with ECL™ chemiluminescent substrate. The exposure lasted for 2 minutes.

Figure 4.22 Experimental design

Strips	1	2	3	4	5
Blocking	5 % skimmed milk in TTBS (0.1 % Tween 20)				
A/B kit	Applied (10 drops each in 5 ml TTBS)				
Serum	AM 1/10	AM 1/10	AM 1/10	Buffer	Buffer
FcεRIα	1/50	1/50	Buffer	1/50	Buffer
Extravidin®	1/2,000	Buffer	1/2,000	1/2,000	1/2,000

AM: atopic dog with *Malassezia* dermatitis.

Results

Binding signals were detected in all strips incubated with Extravidin® (Figure 4.23). The strips incubated with serum exhibited very strong non-specific binding and no distinct bands were visible (lanes 1 and 3). The binding shown by the strip incubated only with serum and Extravidin® (lane 3) indicated that Extravidin® also reacted with certain components in dog serum that might have bound to the blocking reagents non-specifically as this was not seen prior to excessive blocking (Figure 4.19). No improvement was achieved by optimising the dilutions of serum and Extravidin® in a later experiment, and the strips incubated with sera generated similar results (data not shown). General background was seen on the strip incubated only with the IgE receptor and Extravidin® (lane 4) as well as the strip with Extravidin® alone (lane 5). This could stem from insufficient washing during the blotting procedure. Despite this, no bands were detected on these strips, indicating that the blocking had successfully eliminated the cross-reactivity between Extravidin® and the *Malassezia* extract. Nevertheless, the strong non-specific binding shown by the strips incubated with dog serum revealed that this protocol was of no value to detect specific IgE binding proteins in *Malassezia* blots.

In summary, two avidin reagents were tested in protocol 5 and both of them cross-

reacted with *Malassezia* extracts. Although the cross-reactivity of the second reagent, Extravidin[®], was overcome by optimising various reagents as demonstrated in section 4.7.2.2, those conditions did not generate specific binding when used with serum as shown here. This protocol was therefore abandoned due to lack of specificity.

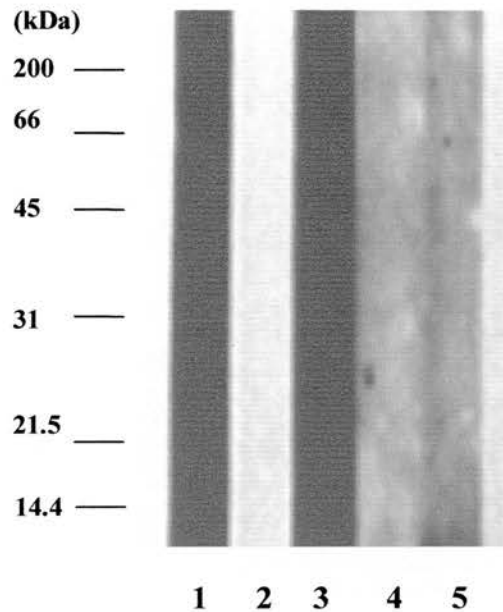


Figure 4.23 Assessment of reagent specificity in *Malassezia* blots using dog sera and detection reagents

Immunoblots showing the results of strips probed with various combinations of reagents after blocking with 5 % skimmed milk in TTBS and 10x avidin/biotin blocking reagents. The strips in lanes 1-3 were incubated with serum from an atopic dog with *Malassezia* dermatitis. The strips in lanes 1, 2, and 4 were then incubated with IgE receptor. Extravidin[®] was applied to all strips except lane 2. The strips that were not probed with working reagents were incubated with buffer alone. Despite the general background which might result from inadequate washing, the lack of bands on the strips incubated without serum, but with Extravidin[®] (lanes 4 and 5) indicated that the cross-reactivity of Extravidin[®] with the *Malassezia* extract had been overcome by the blocking steps. However, the strong non-specific binding shown by the strip incubated with serum and Extravidin[®], but without the IgE receptor (lane 3) suggested a non-specific binding reactivity among the blocking reagents, dog serum, and Extravidin[®].

4.8 Protocol 6–evaluation of a monoclonal antibody/conjugate combination previously used in an ELISA

4.8.1 Cross-reactivity of secondary antibody with canine IgG

Aims

Due to the difficulty in preventing cross-reactivity of the avidin reagents with *Malassezia* proteins in protocol 5, a combination of reagents that had previously been used successfully to detect IgE binding in ELISA assays was assessed to detect IgE binding proteins in the *Malassezia* extract, including a monoclonal anti-dog IgE and an alkaline phosphatase conjugated goat anti-mouse IgG1 antibody that had been passed through a canine IgG column three times. In this protocol, a new detection method was also assessed. A digital image station and compatible software was used to visualise the bands on the blot, rather than overlaying the blot with an X-ray film, as the former provided the advantage of multiple setting options to optimise images. The aim of this experiment was to examine the specificity of the newly purchased AP conjugated goat anti-mouse IgG1 antibody prior to affinity purification.

Methods

Purified mouse IgG1 κ , a normal dog serum sample, and dog IgG were diluted 1/10, 1/100, and 1/20 respectively with PBS and mixed 1:1 with non-reducing buffer before being subjected to electrophoresis. They were then separated in an SDS-PAGE gel and blotted onto a membrane. The membrane was blocked for 1 hour with 5 % skimmed milk in TBS containing 0.1 % Tween 20 (TTBS). After washing with TTBS for 15 minutes (3 x 5 minute rinses), the membrane was incubated for 1 hour with an AP conjugated goat anti-mouse IgG1 antibody (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) diluted 1/1,000 in dilution buffer (1 %

skimmed milk in TTBS). The membrane was washed again in TTBS for a further 15 minutes, and then rinsed with detection buffer comprising 0.1 M Tris-HCl, 0.1 M NaCl, and 5 mM MgCl₂ (BDH, UK), pH 9.5. As the conjugate was labeled with AP, a chemiluminescent substrate for AP labeled reagents, CDP-StarTM (Tropix, Inc., Bedford, USA), was prepared following the manufacturer's instruction and slowly loaded onto the membrane until it was completely covered. After a 5-minute incubation, excess reagents were drained off by holding the membrane vertically against a tissue and the membrane was wrapped in cling film. For visualisation of bands, the membrane was placed on a Kodak Digital ScienceTM Image Station 440CF with the protein side down along with Coomassie blue stained molecular weight markers. The membrane was exposed for 5 minutes, and the blot was visualised on the computer screen.

Results

The unpurified AP conjugated goat anti-mouse IgG1 reacted not only with mouse IgG1 κ , but also normal dog serum and purified dog IgG (Figure 4.24). The conjugate showed binding to the whole molecule of mouse IgG1 as well as its heavy and light chains. Also, the intensity of binding to the whole mouse IgG1 molecule was much stronger than that to the heavy and light chains. The reason for this might be that a minority of the molecules were cleaved during the heating process after mixing with non-reducing sample buffer. However, the binding of the conjugate to normal dog serum and purified dog IgG confirmed its cross-reactivity with dog IgG and the need for further purification.

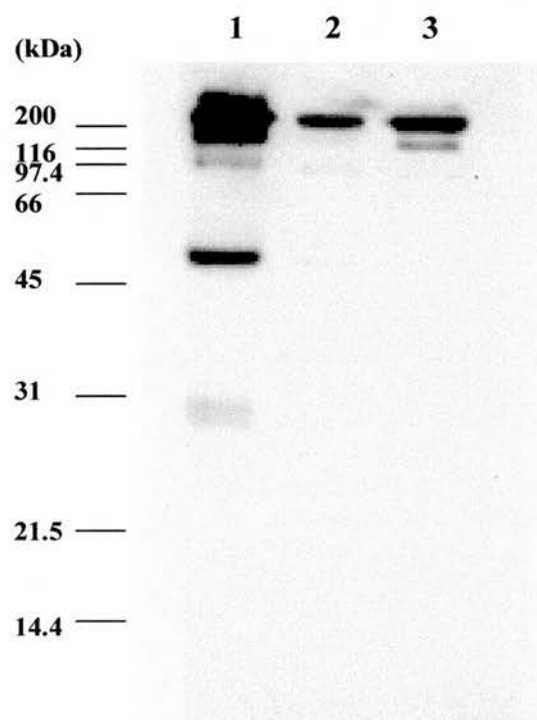


Figure 4.24 Demonstration of the cross-reactivity of the AP conjugated goat anti-mouse IgG1 with canine IgG

Lane1: Mouse IgG1 κ ; Lane 2: Normal dog serum and Lane 3: Purified canine IgG. More than one band was detected in lane 1, likely due to denaturation of the IgG molecule into heavy and light chains. The single band detected in lanes 2 and 3 indicated that the AP conjugated goat anti-mouse IgG1 cross-reacted with purified canine IgG and IgG in dog serum.

4.8.2 Affinity purification of secondary antibody conjugate

Aims

The AP conjugated goat anti-mouse IgG1 was demonstrated to cross-react with canine IgG in the previous experiment. A canine IgG column was therefore used to remove the cross-reactivity of the conjugate by immunoabsorption.

Methods

To eliminate the cross reactivity of the AP conjugated goat anti-mouse IgG1, affinity chromatography was used to further purify the conjugate. Purified dog IgG (10 mg) dissolved in 0.15 M NaCl (Sigma, UK) was exchanged with sodium hydrogen carbonate buffer (0.2 M NaHCO₃ and 0.5 M NaCl, pH 8.5) using HiTrap Desalting (Amersham pharmacia biotech, UK) and coupled to cyanogen bromide (CNBr)-activated Sepharose™ 4B (Amersham pharmacia biotech, UK). The remaining active sites in the Sepharose were blocked with 1M ethanolamine (Sigma, UK), and the gel was then packed into a column. The column was equilibrated using an FPLC® system (Amersham Pharmacia Biotech, UK) with three washing cycles consisting of PBS and citric acid (0.1 M citric acid and 0.5 M NaCl, pH 2.5) for 10 and 3 minutes each at a flow rate of 1 ml per minute, which also helped to flush out unbound residuals. The AP labeled goat anti-mouse IgG1 (Southern Biotechnology Associates, Inc., USA) was then passed through the canine IgG column on three occasions. Antibodies that did not bind to canine IgG were collected. The specificity of the solution passed through the column was checked after each run using the method described in section 4.8.1, and the dilution was carried out by multiplying 1/1,000 by the estimated volume ratio of fluid collected after purification to that loaded into the column on the first run. After the third passage, the purified antibody conjugate was

concentrated using Centriplus™ concentrators (Amicon, Inc., Beverly, USA). The protein concentration of concentrated solution was measured with a spectrophotometer, and diluted 1:1 in glycerol (BDH, UK) to a final concentration of 0.29 mg/ml.

Results

The blots demonstrating the conjugate specificity after each passage through the canine IgG column are shown in Figure 4.25 A-C. After the first passage, the binding of the conjugate to both normal dog serum and purified dog IgG was much weaker compared to Figure 4.24 (Figure 2.25 A), indicating that the majority of the cross-reactivity had been removed. Following passage through the column twice, the conjugate showed even weaker binding to normal dog serum and dog IgG, although it was still detectable using the chemiluminescent detection system (Figure 2.25 B). Elimination of cross-reactivity was seen after the third passage using the concentrated conjugate that had been mixed with glycerol at a dilution of 1/2,000 (Figure 2.25 C). This purified antibody conjugate was therefore used in the subsequent experiments.

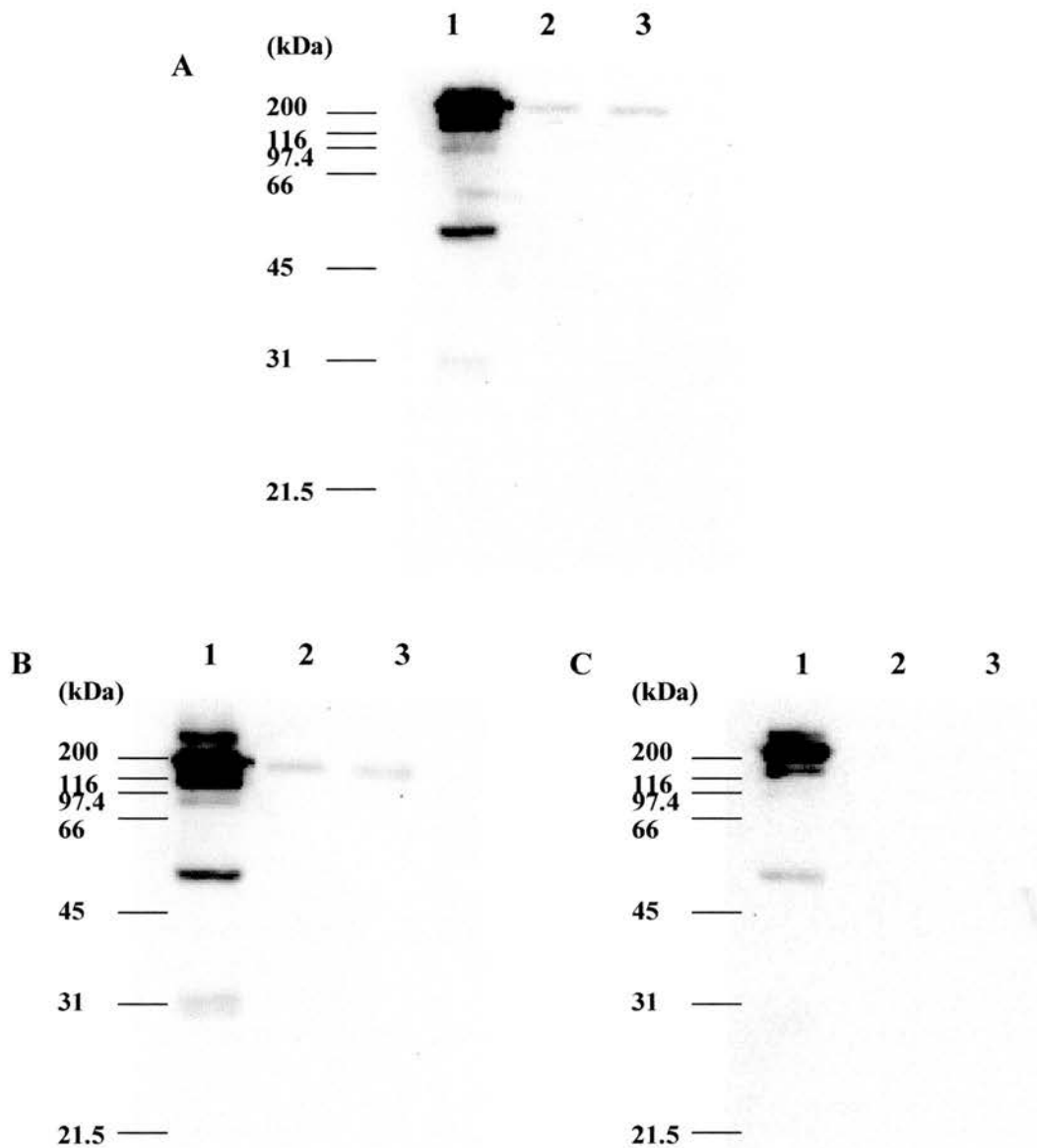


Figure 4.25 A-C Examination of conjugate specificity after purification with affinity chromatography

A: Immunoblot showing the specificity of AP conjugated goat anti-mouse IgG1 that had been passed through the canine IgG column once. The signal in mouse IgG1 κ (lane 1) was very strong, suggesting that the specificity of the conjugate for mouse IgG1 was not affected by purification procedures. However, weak binding to normal dog serum (lane 2) and purified dog IgG (lane 3) indicated that the cross-reactivity was not completely eliminated. B: Immunoblot showing the result after the second passage. The bands in lanes 2 and 3 were slightly weaker than those in A. C: Immunoblot showing the result after the third passage. No bands were detected in lanes 2 and 3 using the concentrated conjugate diluted 1/2,000. This purified conjugate was therefore used in subsequent experiments.

4.8.3 Specificity of the anti-canine IgE antibody and purified conjugate

Aims

The cross-reactivity of the AP conjugated goat anti-mouse IgG1 had been removed by passing the conjugate through the canine IgG column three times as demonstrated in the previous section. However, it was unknown at this stage whether the combination of a monoclonal mouse anti-dog IgE and the conjugate selected in this protocol was specific for canine IgE. The aim of this experiment was to further examine the specificity of these reagents.

Methods

Purified dog IgE and IgG were diluted with PBS, mixed 1:1 with non-reducing sample buffer, and used at a concentration of 0.2 µg per lane. They were then run in an SDS-PAGE gel and transferred to a membrane. The membrane containing separated dog IgE or IgG was blocked for 1 hour with 5 % skimmed milk in TBS containing 0.1 % Tween 20 (TTBS) followed by washing in TTBS for 15 minutes. A monoclonal mouse anti-dog IgE (E6-71A1, Custom Monoclonals International, W. Sacramento, USA) was diluted 1/500 in dilution buffer (1 % skimmed milk in TTBS) and allowed to react with the membrane for 1 hour. For the control groups, the anti-dog IgE antibody was replaced with dilution buffer. After a 15-minute wash, the membrane was incubated for 1 hour with the purified conjugate diluted 1/2,000. It was washed again with TTBS for a further 15 minutes, rinsed with detection buffer, and developed with CDP-StarTM chemiluminescent substrate as described in section 4.8.1.

Results

Binding was only detected on the membrane containing dog IgE with the presence of both monoclonal mouse anti-dog IgE and AP conjugated goat anti-mouse IgG 1 (Figure 4.26). The conjugate did not bind to either dog IgE without the presence of anti-dog IgE, or dog IgG with or without the presence of anti-dog IgE antibody. These results verified that the combination of the monoclonal mouse anti-dog IgE and purified AP conjugated secondary antibody was specific for canine IgE, and these reagents were therefore used in *Malassezia* blots to detect IgE binding proteins from the organism.

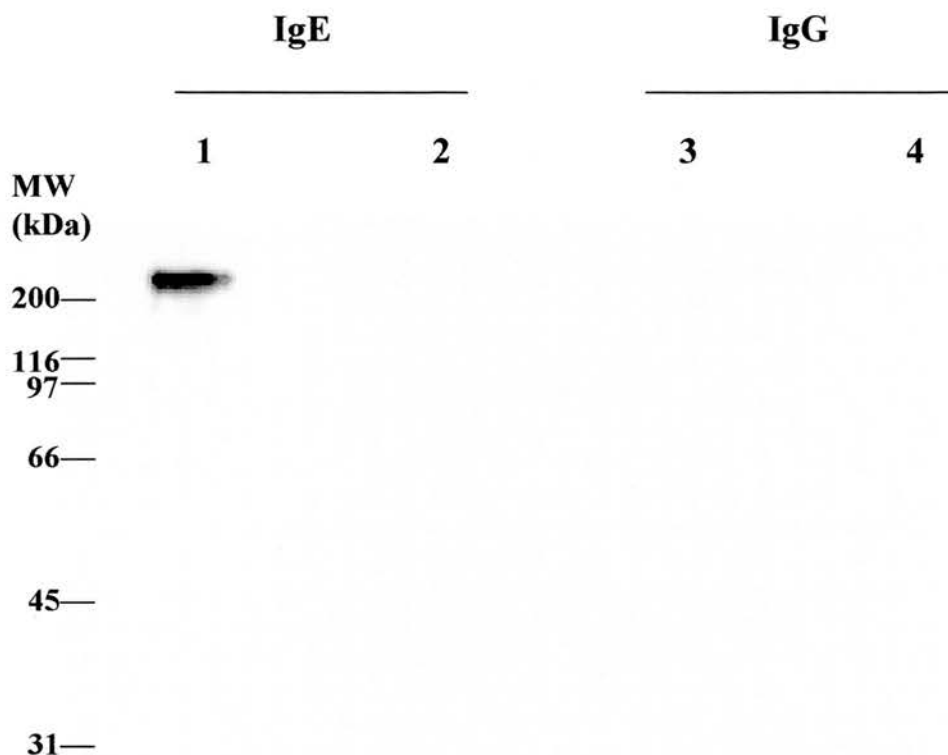


Figure 4.26 Specificity of a monoclonal mouse anti-dog IgE and purified AP conjugated anti-mouse IgG for canine IgE

Purified dog IgE (lanes 1 and 2) and IgG (lanes 3 and 4) were run in an SDS-PAGE gel using non-reducing sample buffer and transferred to a membrane. The membranes were then sequentially incubated with monoclonal mouse anti-dog IgE, goat anti-mouse IgG which had been passed through a canine IgG column three times, and the chemiluminescent substrate (lanes 1 and 3). For the control groups (lanes 2 and 4), the anti-dog IgE antibody was replaced with the dilution buffer. The secondary antibody did not bind to either dog IgE without the presence of anti-dog IgE, or dog IgG with or without the presence of anti-dog IgE antibody.

4.8.4 Optimisation of purified antibody conjugate dilution

Aims

The aim of this experiment was to determine the optimal dilution of AP labeled goat anti-mouse IgG that had been passed through a canine IgG column three times to detect IgE binding components in *Malassezia* blots.

Methods

In chapter 3, IgG binding antigens in *Malassezia pachydermatis* were demonstrated using membranes containing *Malassezia* extract separated in 12 % SDS-PAGE gels. However, 10 % SDS-PAGE gels were deemed to be more suitable for detecting IgE binding proteins in *Malassezia pachydermatis*, as 10 % gels provided better protein separation and more distinct bands on the blot than 12 % gels (data not shown). Therefore, 10 % SDS-PAGE gels were used to separate the *Malassezia* extract in subsequent experiments.

Four strips with separated *Malassezia* proteins were pre-wetted and blocked with 5 % skimmed milk in TTBS for 1 hour. They were washed with TTBS for 15 minutes and then incubated for 1 hour with a serum sample from an atopic dog with *Malassezia dermatitis* diluted 1/5 in dilution buffer (1 % skimmed milk in TTBS). Due to the limited serum supply, only one serum dilution was evaluated in this experiment. Similar serum dilutions to 1/5 had been used to detect IgE binding components from *Malassezia furfur* in man (Johansson & Karlstrom 1991; Jensen-Jarolim *et al.* 1992). The control strip was incubated with dilution buffer alone (Figure 4.27). After further washing in TTBS for 15 minutes, bound IgE was detected by incubating the strips for 1 hour with anti-dog IgE (E6-71A1, Custom

Monoclonals International, W. Sacramento, USA) diluted 1/500 as suggested on the product datasheet. The strips were then washed again in TTBS for a further 15 minutes and incubated with AP conjugated goat anti-mouse IgG diluted 1/2,000, 1/5,000 or 1/10,000 for 1 hour. After another 15-minute wash, the strips were rinsed with detection buffer and incubated with CDP-Star™ chemiluminescent substrate for 5 minutes. Excess reagents were drained off and the strips were wrapped in cling film and exposed for 15 minutes on the Kodak Digital Science™ Image Station. Blots were then visualised on the computer screen.

Figure 4.27 Experimental design

Strips	1	2	3	4
Blocking	5 % skimmed milk in TTBS (0.1 % Tween 20)			
Serum	AM 1/5			Buffer
Anti-IgE	1/500			
2 nd Ab	1/2,000	1/5,000	1/10,000	1/5,000

AM: atopic dog with *Malassezia* dermatitis.

Results

Gradually decreasing binding activity was seen on the strips as the dilution of secondary antibody conjugate was increased (Figure 4.28). Although less background was generated when the conjugate was used at dilutions of 1/5,000 and 1/10,000, the bands on the strips were not as clear as those on the strip incubated with the conjugate diluted 1/2,000. A serum dilution of 1/5, an anti-dog IgE dilution of 1/500, and a conjugate dilution of 1/2,000 were deemed to provide the best results. These conditions were therefore used to analyse individual serum samples.

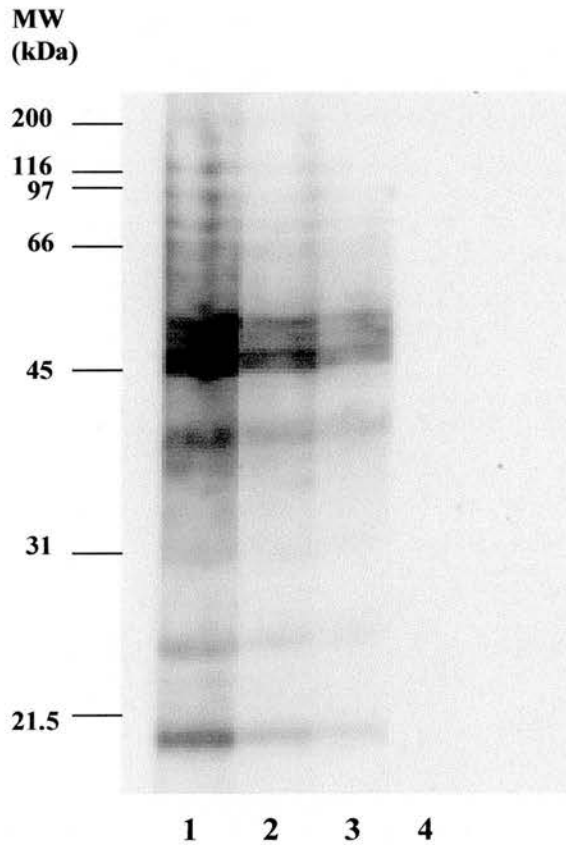


Figure 4.28 Optimisation of goat anti-mouse IgG dilutions

Three different dilutions of AP conjugated goat anti-mouse IgG (1/2,000-1/10,000) were evaluated. The strips were blocked with 5 % skimmed milk in TTBS and incubated with a serum sample from an atopic dog with *Malassezia* dermatitis (lanes 1-3) diluted 1/5. The strip incubated with dilution buffer was used as a control (lane 4). Bound IgE was detected by incubating the strips with anti-dog IgE antibody diluted 1/500 and then anti-mouse IgG diluted 1/2,000 (lane 1), 1/5,000 (lanes 2 and 4) or 1/10,000 (lane 3). Comparing the results from three strips, anti-mouse IgG diluted 1/2,000 was deemed to provide the best outcome and therefore used to analyse all subsequent serum samples.

IgE RESPONSE TO *MALASSEZIA PACHYDERMATIS* ALLERGENS IN DOGS

4.9 Detection of IgE binding proteins from *Malassezia pachydermatis* in atopic dogs with *Malassezia* dermatitis and normal dogs

4.9.1 IgE binding to proteins of *Malassezia pachydermatis* in individual dogs

Aims

In this section, the optimal conditions for immunoblotting to detect *Malassezia pachydermatis* specific IgE were used to analyse individual dog serum samples from atopic dogs with *Malassezia* dermatitis and normal dogs.

Methods

The strips containing separated *Malassezia* proteins were pre-wetted with methanol and blocked for 1 hour with 5 % skimmed milk in TBS containing 0.1 % Tween 20 (TTBS). After washing with TTBS for 15 minutes (3 × 5 minute rinses), each strip was incubated with individual dog serum diluted 1/5 in dilution buffer (1 % skimmed milk in TTBS) for 1 hour. The strips were then washed again in TTBS for a further 15 minutes. Bound IgE was detected by incubating the strips for 1 hour with monoclonal mouse anti-dog IgE E6-71A1 (diluted 1/500). After further washing with TTBS for 15 minutes, the strips were incubated with affinity purified AP-labeled goat anti-mouse IgG1 (diluted 1/2000) for 1 hour. The strips were washed again with TTBS for 15 minutes, and then rinsed with detection buffer. The development was carried out by incubating the strips with CDP-StarTM chemiluminescent substrate for 5 minutes. After draining off excess liquid, the strips were wrapped in cling film and placed on a Kodak Digital ScienceTM Image Station 440CF along with Coomassie blue stained molecular weight markers. The membrane was exposed for 15 minutes, and blots were visualised on the computer screen. Kodak Digital ScienceTM 1D

Image Analysis Software was used to detect the bands on the blots and to determine the molecular weight of bands by analysing their positions relative to molecular weight markers.

Results

The IgE immunoblots of individual dog sera from the two groups are shown in Figure 4.29 A-B. The intensity of binding seen in normal dogs (Figure 4.29 A) was much weaker than that seen in the atopic dogs with *Malassezia* dermatitis (Figure 4.29 B).

The intensity of binding in atopic dogs with *Malassezia* dermatitis was strongest between 45 and 63 kDa. IgE binding proteins with molecular weights of 45 (61 % of dogs), 52 (50 %), 56 (61 %), and 63 (68%) kDa were recognised by more than 50 % of the dogs (Figure 4.30). Strong bands were also detected at 26 (29 %), 48 (39 %), and 60 (18 %) kDa.

Approximately one third of the normal dogs showed faint binding to proteins between 45 and 63 kDa, but clearer bands at 45 (in 18 % of dogs), 52 (36 %), 56 (32 %) and 63 kDa (36 %) were detected by the image analysis software (Figure 4.30). Furthermore, one dog had a prominent band at 26 kDa (No. 9) (Figure 4.29 A).

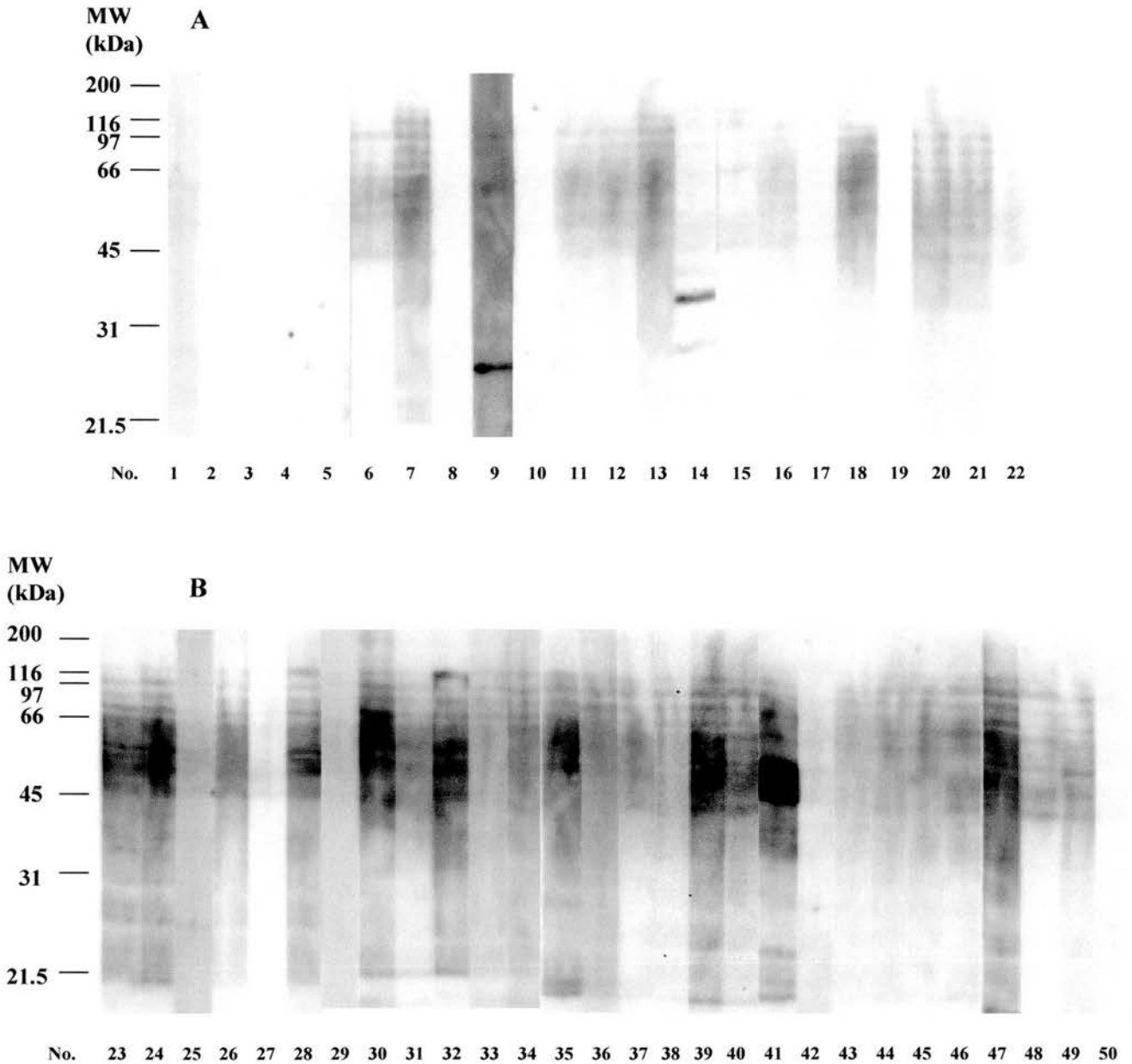


Figure 4.29 IgE-binding proteins in extracts of *M. pachydermatis* detected by immunoblotting with dog sera from two different groups

A: Normal dogs; B: Atopic dogs with *Malassezia* dermatitis. The images of blots were captured by a digital imaging station and printed out directly from the computer. The numbers along the bottom signify the strips probed with individual dog sera. The molecular weight markers on the left are 200, 116, 97, 66, 45, 31, and 21.5 kDa.

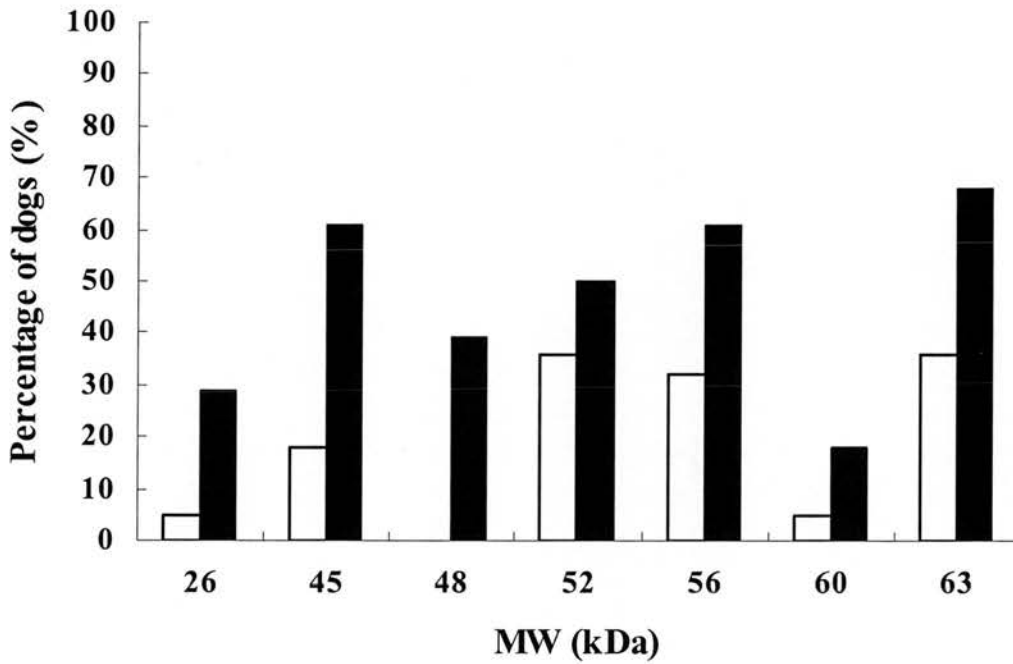


Figure 4.30 Percentage of dogs from the two groups showing IgE binding to individual proteins in extracts of *M. pachydermatis*

The bands on the blots shown in Figure 4.29 were detected using Kodak Digital Science™ 1D Image Analysis Software and the molecular weight of bands generated by the software was used to calculate the percentage of dogs reacting to individual bands. Black columns, atopic dogs with *Malassezia* dermatitis; Open columns, normal dogs. X axis: molecular weights of the bands detected on the strips; Y axis: percentage of dogs reacting to those bands.

4.9.2 Verification of IgE binding

Aims

In order to confirm that the major bands detected on the blots in the previous experiment represented IgE-binding proteins, a pooled serum sample was prepared from the atopic dogs with *Malassezia* dermatitis and from the normal dogs. The pooled serum samples were heat treated at 56°C for 2, 4 and 8 hours to detect the heat lability of binding reactions.

Methods

Two pooled serum samples were generated: one from all the atopic dogs with *Malassezia* dermatitis and one from all the normal dogs. An aliquot of each pooled sample was then heat treated at 56°C for 2, 4 and 8 hours. The immunoblotting was performed on nine pre-wetted strips containing separated *Malassezia* proteins as described in section 4.9.1. After blocking, the strips were incubated with unheated serum pools or pooled serum samples that had been heat treated (Figure 4.31). The strip incubated with dilution buffer (1 % skimmed milk in TBS containing 0.1 % Tween 20) was used as a control. All strips were then analysed with anti-dog IgE and purified secondary antibodies followed by chemiluminescent detection using the same conditions described in section 4.9.1.

Figure 4.31 Experimental design

Strips	1	2	3	4	5	6	7	8	9
Blocking	5 % skimmed milk in TTBS (0.1 % Tween 20)								
Serum 1/5	AM 0	AM 2	AM 4	AM 8	N 0	N 2	N 4	N8	Buffer
Anti-IgE	1/500								
2 nd Ab	1/2,000								

AM: atopic dog with *Malassezia* dermatitis; N: normal dog; 0: non-heated serum pool; 2, 4, and 8: after heating for 2, 4, and 8 hours, respectively.

Results

The binding patterns seen in the two serum pools (Figure 4.32) were very similar to those seen in the corresponding individual serum samples shown in Figure 4.29. As with the individual blots, the binding intensity seen in the atopic dog serum pool was much stronger than that in the normal dogs. Between 45 and 63 kDa, the binding intensity declined dramatically over the period when serum pools were heated. Gradually decreasing binding activity was also seen on one single band at 26 kDa. Accordingly, bands of 26 kDa and any binding detected between 45 and 63 kDa on the strips from the individual dogs were regarded as IgE specific.

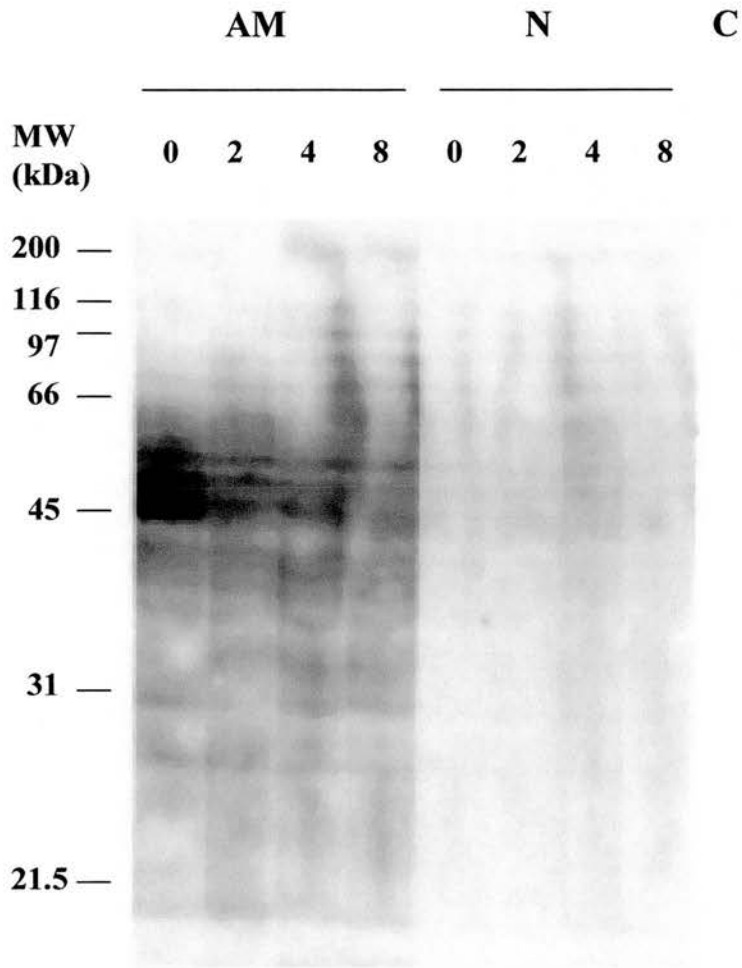


Figure 4.32 Heat-lability of binding detected in two serum pools: one from atopic dogs with *Malassezia* dermatitis and one from normal dogs

AM: atopic dogs with *Malassezia* dermatitis; N: normal dogs; C: control (dilution buffer: 1 % skimmed milk in TTBS). The numbers represent the length of heat treatment. 0: non-heated serum pool; 2, 4, and 8: after heating for 2, 4, and 8 hours, respectively. Note the decrease in staining intensity seen in bands at 26, and 45-63 kDa after heating.

4.10 Discussion

4.10.1 Validation and optimisation of methodology

In the first part of this chapter, six protocols were evaluated to detect IgE binding proteins in *Malassezia pachydermatis*. Whilst optimising the methodology, various problems of lack of sensitivity and specificity were encountered (summarised in Table 4.2). Detection of IgE responses is generally considered to be much more difficult than IgG responses due to the low concentrations of IgE antibodies in sera. The fact that levels of allergen-specific IgG may be several thousandfold higher than levels of IgE in normal and atopic animals (Wassom & Grieve 1998) means that even when there is a small degree of cross-reactivity, the IgG signal may be apparent and interfere with the result. Furthermore, non-specific binding was found between the mixed and complex antigens in the *Malassezia* extract and the secondary-developing reagents. Therefore, various combinations of reagents were evaluated in protocols 1-6 to examine their suitability for use in *Malassezia* immunoblots.

In protocols 1 and 2, a polyclonal anti-dog IgE was used as the primary antibody. Despite the fact that polyclonal antibodies usually provide greater sensitivity than monoclonal antibodies, these two protocols were rejected because there were no visible bands on the blots, even with the amplification step introduced in protocol 2. To increase the sensitivity of the technique, various chemiluminescent protocols were investigated (protocols 3-6). Luminescent detection methods provide several advantages over traditional chromogenic procedures, such as generally increasing the sensitivity of both HRP and AP systems, rapid detection, and long lasting signals permitting multiple exposures (Gallagher *et al.* 1998). To increase the specificity of the chemiluminescent methods, monoclonal antibodies and an IgE receptor reagent

were investigated. Also, the multi-channel miniblotter provided several potential advantages over cutting membranes into strips, and its use in *Malassezia* blots was evaluated (section 4.5.1). In protocols 3 and 4, the secondary antibodies cross-reacted with canine IgG and they were abandoned due to lack of specificity. This problem is confounded by the fact that levels of *Malassezia*-specific IgG are much higher than levels of IgE as shown by the results obtained in chapter 3 using a less sensitive substrate (DAB) to detect *Malassezia*-specific IgG. The high levels of *Malassezia*-specific IgG in dog sera could enhance the IgG signals and eclipse the IgE reaction.

The high-affinity receptor for IgE, FcεRI, is a requisite for the IgE-mediated immediate hypersensitivity reaction and the interaction between IgE and FcεRI is highly specific (Turner 2001). FcεRI has been used in ELISAs to measure canine IgE because of its high affinity for canine IgE and lack of reactivity with canine IgG (Wassom & Grieve 1998; Stedman *et al.* 2001). In view of its likely ability to eliminate the problems of cross-reactivity with canine IgG as seen in protocols 3 and 4, the suitability of the receptor for use in *Malassezia* blots was investigated in protocol 5. In spite of offering absolute specificity for IgE, the detection with FcεRI receptor reagent was not successful because the secondary avidin reagents bound to *Malassezia* proteins. As the *Malassezia* extract used in this study contained a crude mixture of both cytoplasmic and cell wall components, the non-specific binding between avidin reagents and *Malassezia* proteins was likely to stem from endogenous protein-bound biotin in the extract. This was further confirmed by optimising various reagents to abolish the cross-reactivity including avidin/biotin blocking solutions (section 4.7.2.2).

In protocol 6, a combination of reagents that had previously been used successfully to detect IgE binding in ELISA assays (unpublished observations) was assessed. In this protocol, the cross reactivity of the secondary antibody was removed by immunoabsorption through a canine IgG column. Although protocol 6 proved to be successful, it is likely that the use of the cross-absorbed secondary reagent with other primary antibodies would also be suitable. With this protocol, a digital imager was used to resolve the bands, rather than assessment of X-ray films. It was found that x-ray films produced previously reported problems such as weak bands being overshadowed by adjacent strong signals, broad diffuse images resulting from overexposure and film saturation. The image station and image analysis software provided better resolution of the bands and enabled more accurate determination of their molecular weights than traditional chemiluminescent methods. It is important to note that chemiluminescent blots are not visible to the naked eye and can only be visualised on X-ray film or on a computer screen (when using digital imaging). Furthermore, the bands on chemiluminescent blots are often not as clear and distinct as those seen on normal chromogenic blots. However, the use of imaging software allows the bands to be resolved without investigator bias.

Despite the various problems associated with lack of specificity, there are three lines of evidence that confirm *Malassezia*-specific IgE has been detected in the present study. First, it was demonstrated that the reagents used in protocol 6 did not cross-react with canine IgG (see Figure 4.26). Secondly, the IgE binding to the major allergens was shown to be heat labile (Figure 4.32). Thirdly, the antigens detected in this chapter were different from those detected in chapter 3 using reagents to detect IgG.

4.10.2 IgE response to *Malassezia pachydermatis* allergens in dogs

In the second part of this chapter, specific IgE binding proteins in extracts of *Malassezia pachydermatis* were demonstrated using sera from atopic dogs with *Malassezia* dermatitis and normal dogs. The results of the immunoblotting with individual dog sera indicate that the majority of the atopic dogs with *Malassezia* dermatitis had a greater IgE response to allergens of *M. pachydermatis* than normal dogs suggesting that *Malassezia* dermatitis in atopic dogs is associated with an IgE response to the organism. This also confirms the findings of previous studies that have demonstrated elevated concentrations of *Malassezia*-specific IgE in atopic dogs with *Malassezia* overgrowth by ELISA (Nuttall & Halliwell 2001) and positive responses following intradermal injection of *Malassezia* extracts (Morris *et al.* 1998; Bond *et al.* 2002a). Furthermore, the immunoblots showed that the IgE response was predominantly targeted against proteins with molecular weights of 45, 52, 56, and 63 kDa. As these proteins were recognised in over 50 % of the atopic dogs with *Malassezia* dermatitis, but only recognised by a minority of normal dog sera, they can be classified as major allergens. Strong bands were also detected with molecular weights of 26, 48, and 60 kDa but as these were recognised in less than half of the atopic dogs, they can be regarded as minor allergens. Weak IgE binding was also seen in some normal dogs between 45 and 63 kDa. The cause of this weak IgE binding in normal dogs is unknown. It has been shown that extracts of *M. pachydermatis* at high concentrations are capable of inducing wheal and flare responses in normal dogs (Morris *et al.* 1998). This could be an irritant reaction or an IgE response present to commensal populations of the yeast. The latter is certainly the case with IgG responses as shown in previous studies (Chen *et al.* 2002; Nuttall & Halliwell 2001). It has also been proposed that the positive IDTs to environmental

allergens and the presence of allergen-specific IgE in normal dogs might be explained by the existence of heterogeneity of IgE or variations in the propensity for mediator release between atopic and normal dogs (Halliwell *et al.* 1998; Halliwell & DeBoer 2001). This is speculated from the evidence that human IgE may be functionally heterogeneous in terms of its ability to mediate histamine release (MacDonald *et al.* 1995), the finding of physico-chemical heterogeneity in canine IgE (Peng *et al.* 1997) and the tendency of leukocytes from atopic dogs to release more histamine than those from normal dogs (Jackson *et al.* 1996).

Of all the allergens detected, those with molecular weights of 45 and 48 kDa seemed to be the most relevant because they were recognised by most atopic dogs with *Malassezia* overgrowth but only a few normal dogs (Figure 4.30). Further studies are required to determine the identity of these proteins using protein purification and N-terminal sequencing. They may represent two different proteins or a single protein with variable glycosylation.

In contrast to the low molecular weight allergens of *Malassezia furfur* (Mal s 1, Mal f 2–Mal f 4, Mal s 5–Mal s 9) recognised by humans, dogs recognised higher molecular weight allergens in this study. Similar findings have been documented with *Dermatophagoides* allergens. Most human atopic sera have high IgE titers to group 1 and group 2 allergens of *Dermatophagoides* (Le Mao *et al.* 1998; Thomas *et al.* 1998) which are low molecular weight proteins, whereas in dogs, the most important *Dermatophagoides* allergens are high molecular weight proteins greater than 90 kDa (Noli *et al.* 1996; McCall *et al.* 2001; Nuttall *et al.* 2001). Although a few *Malassezia* proteins in the molecular weight range between 46 and 58 kDa were

recognised by more than 50 % of the human atopic patients in one study (Huang *et al.* 1995), similar to the results obtained in the present study, their characteristics have not been further defined.

In human studies, it is considered that both protein and carbohydrate antigens of *Malassezia* spp. are important, although the importance of carbohydrate antigens is emphasised less, probably due to their appearance as diffuse bands or even absence in SDS-PAGE gels (Ashbee & Evans 2002). In addition to the proteins with various molecular weights identified by immunoblotting, one study reported positive RAST results with *Malassezia* mannan in 77 % of the patients with atopic dermatitis indicating that mannan was also an important allergen of *Malassezia* organisms colonised on human skin (Lintu *et al.* 1997). It has been shown that the cross-reacting IgE antibodies between *Malassezia* spp. and *Candida albicans* were likely to be directed at mannans or mannoproteins (Savolainen & Broberg 1992; Doekes *et al.* 1993; Doekes & Ieperen-van Dijk 1993). Although a correlation between *M. pachydermatis* extracts and mold mix allergens was not found in dogs (Morris *et al.* 1998), the importance of carbohydrate antigens in dogs is uncertain and awaits further investigation.

In summary, the experiments in this chapter have developed and validated a Western blotting technique to detect specific IgE binding proteins from *M. pachydermatis*. Using this technique, proteins of 45, 52, 56, and 63 kDa were demonstrated to be recognised by over 50 % of the atopic dogs with *Malassezia* dermatitis and appeared to be clinically relevant in these dogs. However, further characterisation of these allergens with protein purification and sequencing techniques is required before they

could be used for diagnosis and treatment purposes. The purified allergens could potentially be used in intradermal testing and *in vitro* IgE assays to provide additional diagnostic information or in allergen immunotherapy to benefit affected dogs.

Chapter 5

THE EFFECT OF EXTRACTS FROM *MALASSEZIA PACHYDERMATIS* ON THE PROLIFERATION OF CANINE KERATINOCYTES

5.1 Introduction

Malassezia dermatitis, like a number of other chronic inflammatory dermatoses, is often associated with marked epidermal hyperplasia (Scott & Miller 1989; Yager 1994; Mauldin *et al.* 1997; Muse 2000; Scott *et al.* 2001a). Epidermal hyperplasia is a protective mechanism mounted by the skin in response to environmental insults and is caused by an increase in the proliferation of keratinocytes resulting in increased number of epidermal cells (Scott *et al.* 2001d). The regulation of keratinocyte proliferation is an extremely complex process and the molecular mechanisms involved are of great clinical importance in terms of understanding the pathogenesis and treatment of skin diseases. The essential mechanism by which cells divide is a cycle of duplication and division, also known as the cell cycle, which comprises four phases including M, G1, S, and G2 phases (Alberts *et al.* 2002a). In keratinocytes, the cell cycle is mainly regulated in the G1 phase and the progression through the cycle is determined by the stage of cell differentiation, cell adhesion, and the balance between stimulatory and inhibitory growth factors (Gniadecki 1998). The cells that have proliferative capacity in the epidermis are located in the basal layer, whereas terminally differentiated suprabasal keratinocytes are unable to proliferate (Suter *et al.* 1997; Gniadecki 1998; Hashimoto 2000). Adhesion to a substratum, such as tissue culture plastic or purified extracellular matrix proteins, is known to be

required for keratinocyte growth, and loss of attachment can cause a rapid growth arrest (Gniadecki 1998). It has also been shown that cell attachment is important for expression of cyclins and complex formation between cyclin and cyclin-dependent kinases (cdks) in the G1 phase, both of which play pivotal roles in controlling the cell proliferation cycle (reviewed in Assoian 1997).

5.1.1 Stimulatory factors for keratinocyte proliferation

5.1.1.1 Epidermal growth factor family

Growth factors are typically secreted proteins that are released from cells, act upon their target, and regulate cell proliferation (Heath 2001). Among the factors that stimulate keratinocyte growth, the epidermal growth factor (EGF) family is of central importance (Suter *et al.* 1997; Hashimoto 2000). The EGF family comprises EGF itself, transforming growth factor (TGF)- α , heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), epiregulin, betacellulin (BTC), and neuregulins. TGF α , HB-EGF, AR and epiregulin are produced and secreted by human keratinocytes (Hashimoto 2000; Heath 2001). Stimulation of keratinocytes with EGF and TGF α results in an increased rate of proliferation and migration (Barrandon & Green 1987; Gibbs *et al.* 2000). HB-EGF and AR, which have binding affinity for heparinoids, in addition to TGF α and epiregulin, act as autocrine growth factors and form a regulatory network during keratinocyte growth (Piepkorn *et al.* 1994; Hashimoto *et al.* 1994; Shirakata *et al.* 2000).

EGF receptors include four forms (ErbB1-4, also referred to as Her1-4), of which EGF, HB-EGF, TGF α , AR, BTC and epiregulin bind to ErbB1, whereas neuregulins bind to ErbB3 and 4 (Hashimoto 2000). The binding of a ligand to the receptor

causes the receptor to dimerize and activate intracellular protein tyrosine kinases, which then bind to adaptor proteins such as Shc, Grb2, and Sos, leading to the activation of Ras protein (Tomic-Canic *et al.* 1998; Freedberg *et al.* 2001). Activated Ras, in turn, activates a cascade of three protein kinases, Raf1, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) (MEK), and ERK, the last of which translocates to the nucleus, phosphorylates, and activates transcription factors, such as ATF2, SAP1, c-Jun, and Elk1. These transcription factors then regulate gene expression and cell division (Freedberg *et al.* 2001).

5.1.1.2 Fibroblast growth factor family

As with the EGF family, the fibroblast growth factor family comprises several structurally related mediators which currently include 16 members (Werner 1998). The initially discovered acidic fibroblast growth factor (aFGF) and basic FGF (bFGF) can both be synthesized by keratinocytes (Feliciani *et al.* 1996) and are now called FGF 1 and 2. The latter is more potent at stimulating the growth of keratinocytes (Rothe & Falanga 1989; Suter *et al.* 1997). Another member of the FGF family, keratinocyte growth factor (KGF, FGF-7), is produced by fibroblasts and has a role in wound repair in which it influences proliferation and/or differentiation of keratinocytes (Werner 1998; Werner & Smola 2001). The actions of FGFs on cells are mediated via binding of three different molecules: heparan sulfate proteoglycan, a low-affinity transmembrane FGF-binding protein, and a high affinity transmembrane FGF receptor of the tyrosine kinase family (Werner 1998; Alberts *et al.* 2002b). Four FGF receptors (FGFR 1-4) have been described and the different members of the FGF family bind with differing affinity, although KGF only binds to a splice variant of FGFR-2 (Williams & Kupper 1996; Werner 1998). Binding of FGF leads to

dimerization of FGF receptors, activation of receptor tyrosine kinases, and subsequent autophosphorylation of the cytoplasmic domains (Klint & Claesson-Welsh 1999). The autophosphorylation sites serve as binding sites for enzymes, such as phospholipase C- γ and Src, as well as adaptor proteins, Shc, Grb2, and Sos. The binding signals then trigger activation of the Ras signaling pathway leading to up-regulation of ERKs and cell proliferation as described above for the EGF family (Boilly *et al.* 2000).

5.1.1.3 *Insulin-like growth factors*

Insulin-like growth factor 1 (IGF-1), one of two members currently known in this family, has been identified as a keratinocyte mitogen (Neely *et al.* 1991; Barreca *et al.* 1992). IGF-1 is produced by keratinocytes of the stratum granulosum and by dermal fibroblasts (Rudman *et al.* 1997; Suter *et al.* 1997). The stimulatory effects of IGF-1 on keratinocytes are associated with its interaction with receptors for EGF and induction of TGF α and amphiregulin (Krane *et al.* 1991; Vardy *et al.* 1995). IGF-1 receptors are localised to the basal proliferative cells in the normal epidermis (Krane *et al.* 1991). Three survival (anti-apoptosis) signaling pathways have been demonstrated for IGF-1 receptors (Navarro & Baserga 2001). However, the pathways that signal cells to proliferate have not been fully elucidated. The most well-known mitogenic/survival pathway is via the activation of phosphatidylinositol-3 kinase (PI3K). The cytoplasmic tyrosine kinase domains of IGF-1 receptors undergo autophosphorylation upon IGF-1 stimulation, which then phosphorylate a substrate of the IGF-1 receptor, insulin receptor substrate-1 (IRS-1), thus activating PI3K. PI3K, in turn, activates protein kinase B which inactivates BAD, a protein that normally encourages cells to undergo apoptosis, thereby promoting cell survival

(Alberts *et al.* 2002b).

5.1.1.4 Interleukin 1 (IL-1)

IL-1 is an initiator of keratinocyte activation and its characteristics and functions have been extensively reviewed (Sauder 1989; Freedberg *et al.* 2001; Grone 2002). Two major forms of IL-1, α and β , are present in an unprocessed form in the cytoplasm of keratinocytes (Freedberg *et al.* 2001). In response to injury, keratinocytes process and release IL-1, which serves as an autocrine signal, resulting in activation and proliferation (Kupper 1990). Keratinocytes express three different receptors for IL-1: type I receptor (functional), type II receptor (decoy), and IL-1 receptor antagonist (Eller *et al.* 1995; Feliciani *et al.* 1996). After IL-1 binds to type I receptors on the cell surface, the intracellular domain of the receptor recruits protein kinases leading to activation of at least three transcription factor systems in the nucleus, resulting in modulation of gene expression (Freedberg *et al.* 2001).

5.1.1.5 Interleukin 6 (IL-6)

IL-6 is produced in small quantities by normal keratinocytes, but production increases after stimulation with cytokines such as IL-1 and TNF α , and physical insults such as ultraviolet light exposure (Feliciani *et al.* 1996; Suter *et al.* 1997; Grone 2002). The induction of keratinocyte proliferation by IL-6 has been demonstrated *in vivo* (Sawamura *et al.* 1998; Sato *et al.* 1999). Binding of IL-6 to its receptor at the cell surface causes the activation of cytoplasmic tyrosine kinases, Jak1/Jak2/Tyk2, which then phosphorylate a gene regulatory protein, signal transducers and activators of transcription-3 (STAT3). When phosphorylated, STAT3 conveys the signal directly from the plasma membrane into the nucleus without

second messengers or cytoplasmic kinase cascade intermediates. It then dimerises and translocates to the nucleus where it activates gene transcription (Tomic-Canic *et al.* 1998).

5.1.1.6 Granulocyte macrophage-colony stimulating factor (GM-CSF)

Keratinocytes produce large quantities of GM-CSF following injury or stimulation with tumor promoters (Feliciani *et al.* 1996; Mann *et al.* 2001). GM-CSF was recently shown to induce both keratinocyte proliferation and apoptosis *in vivo* (Breuhahn *et al.* 2000). As a result, transgenic mice with constitutive overexpression of GM-CSF by keratinocytes in unwounded skin showed normal epidermal thickness and differentiation indicating that GM-CSF alone did not evoke epidermal hyperplasia (Breuhahn *et al.* 2000). Based on the same model, a further study proposed that the accelerated process of wound healing seen in these transgenic mice was due to not only the direct action of GM-CSF, but also the indirect effect of its induction of other cytokines (Mann *et al.* 2001). The kinase associated with the GM-CSF signaling is Jak2, which then activates STAT5 (Alberts *et al.* 2002b).

In addition to the growth factors and cytokines listed above, other factors that have been associated with induction of keratinocyte proliferation include human leukocyte elastase (Rogalski *et al.* 2002), nerve growth factor (Pincelli & Marconi 2000), histamine and serotonin released by mast cells (Maurer *et al.* 1997), and urokinase-type plasminogen activator (uPA) (Jensen & Lavker 1999).

5.1.2 The effects of microorganisms on keratinocytes

Following infection with microorganisms, there is some evidence that suggests

keratinocytes play an active role in host defense mechanisms and the pathogenesis of dermatitis. One study investigated the production of proinflammatory molecules produced by keratinocytes co-cultured with *Streptococcus pyogenes*. Infection of keratinocytes with adherent, but not nonadherent, streptococci resulted in increased expression of mRNA for IL-1 α , IL-1 β , and IL-8 (Wang *et al.* 1997). Both adherent and nonadherent streptococci induced expression of IL-6 and promoted release of prostaglandin E₂, but adherent streptococci were much more potent (Wang *et al.* 1997). Stimulation of TNF α production by keratinocytes has also been reported following incubation with Staphylococcal enterotoxin B (Tokura *et al.* 1994). Similar studies have also been performed with *Malassezia* organisms. In contrast to streptococci, *Malassezia furfur* down-regulated mRNA expression for IL-1 α , inhibited the expression of IL-6 and TNF α , and up-regulated the immunosuppressive cytokines IL-10 and TGF- β 1. These findings may explain the minimal inflammation seen in the human dermatosis associated with *M. furfur* (Baroni *et al.* 2001a). The release of IL-1 α by normal human keratinocytes in response to extracts from *M. furfur* serovar B (*M. globosa*) was reported to be donor-dependent (Walters *et al.* 1995). The release of IL-1 β , IL-6, IL-8 and TNF α from normal human keratinocytes co-cultured with *M. furfur*, irrespective of whether the whole organisms or culture supernatants were used, was low or undetectable (Watanabe *et al.* 2001). However, infection of human keratinocytes with whole organisms of *M. pachydermatis*, *M. slooffiae* and *M. sympodialis*, but not culture supernatants, was shown to induce secretion of IL-1 β , IL-6, IL-8 and TNF α , with the highest levels seen with *M. pachydermatis* (Watanabe *et al.* 2001).

Epidermal proliferation has been implicated as an important mechanism in the

defense against superficial fungal infections. Increased proliferative activity and thickness of the epidermis has been demonstrated in mice/guinea pigs with experimental dermatophytosis and candidiasis (Sohnle & Kirkpatrick 1978; Tagami 1985; Sohnle & Hahn 1989). In contrast to the epidermal responses to fungi, one study showed no direct effect of M proteins, receptor proteins of *S. pyogenes*, on the proliferative activity of human keratinocytes (Wollina *et al.* 1998). To the author's knowledge, no similar study has been reported in human keratinocytes co-cultured with *Malassezia* organisms, but a preliminary report demonstrated a significant increase in a cellular proliferation marker in cultured canine keratinocytes infected with *M. pachydermatis* compared to non-infected cells (von Tscharnher *et al.* 1999). However, the mechanisms by which *M. pachydermatis* might induce keratinocyte proliferation are not understood.

5.1.3 Aims

One hypothesis for the epidermal hyperplasia seen in *Malassezia* dermatitis is that a product within, or secreted from, the organism may have a direct stimulatory effect on keratinocyte proliferation. The aim of this study, therefore, was to test this hypothesis by evaluating the direct effects of extracts and culture supernatants from *Malassezia pachydermatis* on the proliferation rate of cultured canine keratinocytes. The initial purpose of the experiments was to evaluate the use of a colourimetric cell proliferation assay to detect the number of viable canine keratinocytes. The assay was then used to quantify the number of keratinocytes cultured with extracts and culture supernatants from *M. pachydermatis*.

VALIDATION AND OPTIMISATION OF A CELL PROLIFERATION ASSAY TO DETECT KERATINOCYTE PROLIFERATION

5.2 Correlation between cell numbers and absorbance in the assay

Aims

In order to use a colourimetric cell proliferation assay to quantify the number of canine keratinocytes, it was essential to demonstrate that the absorbance was proportional to the number of living keratinocytes in culture. The aim of the initial experiment was to validate the feasibility of using the cell proliferation assay with canine keratinocytes and to determine the optimal assay conditions.

Methods

Keratinocytes grown in tissue culture flasks were harvested by trypsinisation (section 2.19). After the cells were washed and counted in a haemocytometer (section 2.17), 100 µl of cell suspension (containing two fold dilutions from 2×10^5 to 3125 viable cells per well) was added in triplicate to a 96-well plate. Wells with 100 µl of medium only were included as background controls. Twenty microliters of Celltiter 96[®] AQueous One Solution reagent (Promega, Southampton, UK) was then added to all wells and the plate was incubated at 37°C in a humidified atmosphere with 5 % CO₂ in air for up to 4 hours (an incubation time of 1-4 hours was suggested by the manufacturer). In order to determine the optimal incubation time for canine keratinocytes, the absorbance of the reaction was evaluated after incubation for 2, 3 and 4 hours using an ELISA reader (Dynatech MR5000, Dynatech Laboratories Ltd, Billingshurst, UK) at a wavelength of 490 nm and a reference wavelength of 630 nm (section 2.25). The plate was returned to the incubator for further colour development between each reading.

Results

The absorbance obtained after incubation for 2, 3, and 4 hours was plotted against the number of viable cells per well. A linear correlation was found between the absorbance and cell number when both parameters were transformed to logarithms (Figure 5.1 A-C). This assay was therefore judged to be a valid method for measuring canine keratinocyte proliferation *in vitro*.

The absorbance obtained after a 2-hour incubation (Figure 5.1 A) was the lowest among three sets of results. Higher absorbance was seen after a 3-hour incubation (Figure 5.1 B), and there was only a small increase of absorbance when the plate was incubated for a further 1 hour (Figure 5.1 C). These results indicated that a 3-hour incubation was adequate for colour development and was thus used for subsequent experiments.

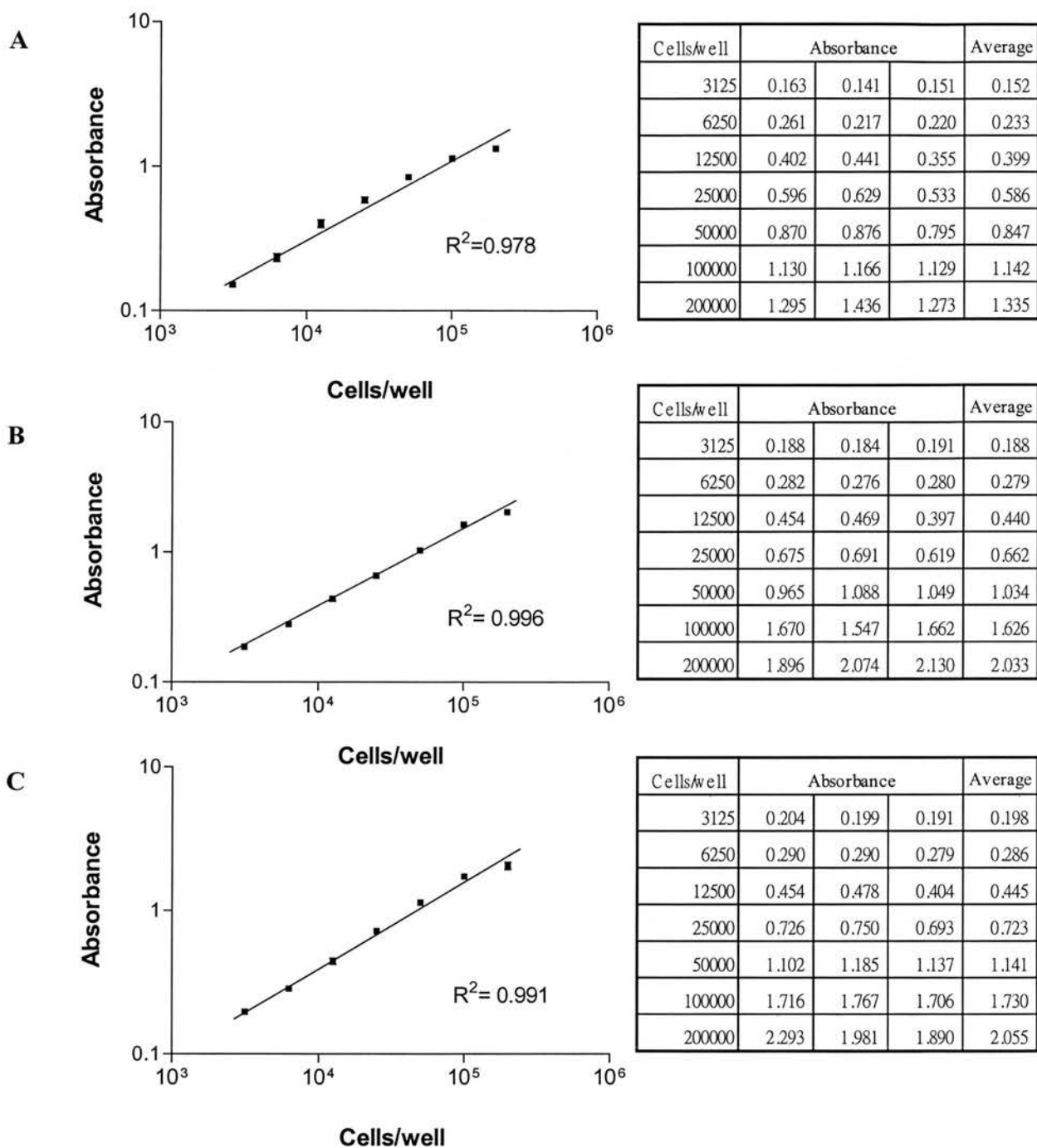


Figure 5.1 A-C Correlation between cell numbers and absorbance in the Celltiter 96 colourimetric assay

A: Results obtained after incubation for 2 hours. B: After 3 hours. C: After 4 hours. The absorbance is proportional to cell number between 2×10^5 and 3125 when both X and Y axes are transformed to logarithmical scales. Higher absorbance was seen when the incubation time was increased. However, little difference was observed between the results obtained after 3 and 4 hours. Data shown are the mean of triplicates, but the error bars are so small they cannot be displayed on the figure. X axis: cell number per well, Y axis: absorbance at 490 nm with a reference wavelength of 630 nm.

5.3 Effects of growth factors on keratinocyte proliferation

Aims

To verify that the culture conditions used to grow keratinocytes (section 2.16) were optimal and to further confirm that the colourimetric cell proliferation assay was suitable to evaluate keratinocyte growth.

Methods

Cells harvested from tissue culture flasks were washed, resuspended in William's Medium E (WME) and counted in a haemocytometer. Various combinations of supplements including foetal calf serum (FCS), cholera toxin (CT), and murine epidermal growth factor (mEGF) were added to the concentrations of 10 %, 0.1 nM, and 10 ng/ml, respectively. Cells were then plated into a 96-well plate in quadruplicate (100 μ l/well, 2×10^4 cells/cm²), and cultured in WME alone; with WME and FCS; with WME, FCS and CT; with WME, FCS and mEGF; and with WME, FCS, CT, and mEGF (complete medium). The density of cell plating was determined in preliminary experiments (data not shown). Three identical plates were prepared for performing the assay on three different days and the plates were cultured at 37°C with 5 % CO₂ in air for up to 7 days. Cells from the same passage were also plated in flasks to be used for generating a standard curve in the cell proliferation assay.

The proliferation assay was carried out on days 3, 5, and 7. Medium in the remaining plates was changed accordingly on the same days. On the day of the assay, cells grown in the flasks were trypsinised, washed and counted in a haemocytometer to generate a standard curve. A volume of 100 μ l of cell suspension (containing two

fold dilutions from 2×10^5 to 3125 cells per well) was added in triplicate to empty wells. Wells used for background controls were filled with 100 μ l of medium alone. Twenty microliters of Celltiter 96[®] AQueous One Solution reagent was then added to all the wells and the plate was incubated for a further 3 hours before evaluating the absorbance of the reaction using an ELISA reader. The absorbance of wells containing keratinocytes and WME with or without various combinations of supplements was compared with the standard curve and the results were calculated as cells per well, all of which were generated automatically by the ELISA reader. This experiment was repeated on three different occasions.

Results

The results obtained from three repeated experiments were very similar and a representative set of results obtained from three assays is shown in Figure 5.2. Cells grown in the complete medium (containing FCS, CT, and mEGF) had the highest proliferation rate, with more cells detected than in the other groups in all three assays performed during the seven-day incubation. This contrasted with the cells cultured in WME alone, showing minimal increase in number on days 3 and 5, and a discontinuation of growth on day 7. Although higher growth rates were seen in the groups with addition of FCS, FCS plus CT, and FCS plus mEGF, they fell between those of the previous two groups. Hence, canine keratinocyte cultures were routinely grown in complete medium since it provided the best growth rates. Also, the results of this section further confirmed the validity of the cell proliferation assay to quantify canine keratinocytes.

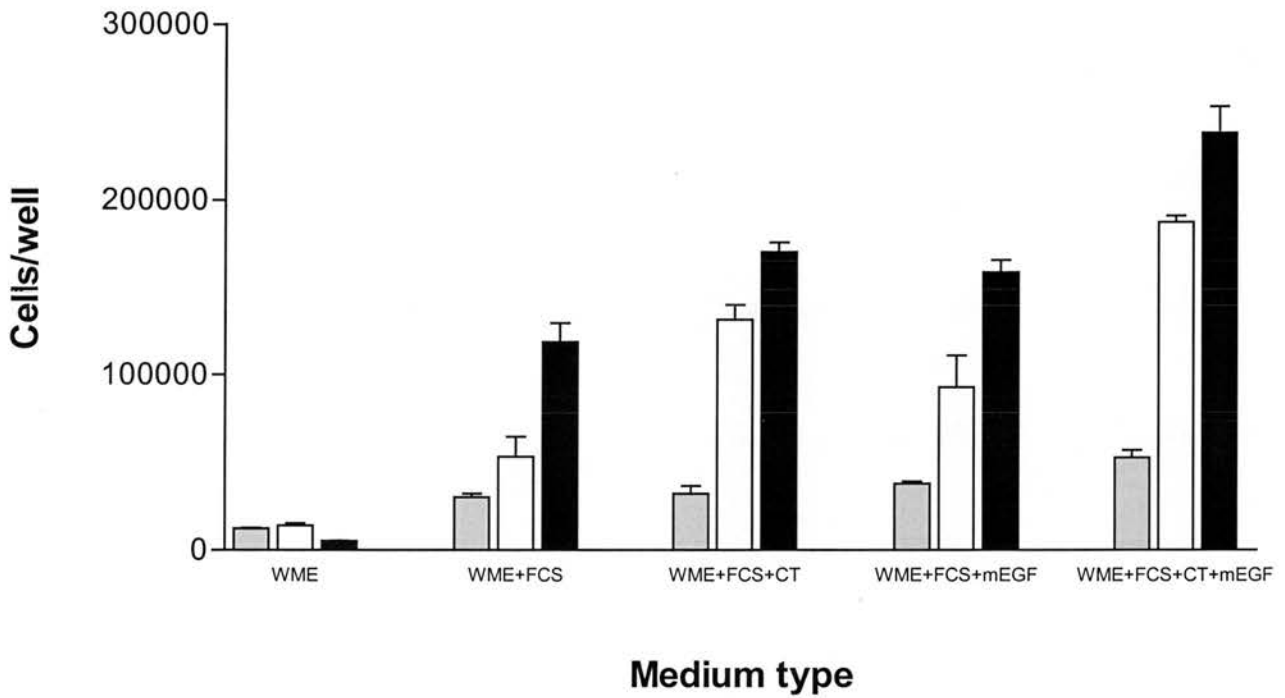


Figure 5.2 Comparison of the proliferation rate of keratinocytes cultured in William's Medium E with and without supplementation

The data set shown is a representative example of three repeated experiments giving similar results. Keratinocytes grown in medium with FCS, CT and mEGF (complete medium) showed the highest growth rate compared to the other groups and this was detected in all three assays carried out on days 3, 5 and 7. In contrast, unsupplemented WME failed to support keratinocyte growth. Therefore, keratinocytes were routinely cultured in complete medium. Each bar represents the mean and standard error of the mean of quadruplicates. Shaded bars: day 3, White bars: day 5, Black bars: day 7. WME: William's medium E; FCS: foetal calf serum; CT: cholera toxin; mEGF: murine epidermal growth factor.

**EFFECTS OF EXTRACTS FROM *MALASSEZIA PACHYDERMATIS* ON
THE PROLIFERATION OF CANINE KERATINOCYTES**

5.4 Culture of keratinocytes with extracts from *Malassezia pachydermatis*

**5.4.1 Establishment of assay conditions for culture of keratinocytes with
Malassezia extracts**

Aims

The aim of this experiment was to determine the concentration range of *Malassezia* extracts used for co-culture with keratinocytes and the optimal time for performing the proliferation assay.

Methods

Cultured keratinocytes were washed, resuspended in complete medium and counted in a haemocytometer. Some cells were plated into flasks to be used for generating a standard curve in the assay and the others were plated into two 96-well plates at a density of 2×10^4 cells/cm² (100 µl/well). The plates were incubated at 37°C for 24 hours to allow cells to attach to the plastic, and the medium was changed the next day to remove non-adherent cells. Sterile *Malassezia* extracts (see section 2.7) prepared with protease inhibitors (10 mg/ml) were diluted 10-fold with PBS and 100 µl of each dilution was added in quadruplicate into wells containing keratinocytes and 100 µl of fresh medium, resulting in final concentrations of 5, 5×10^{-1} , 5×10^{-2} , 5×10^{-3} , 5×10^{-4} and 5×10^{-5} mg/ml. Wells containing medium alone and PBS served as controls. After a further 3 day incubation, one plate was used for the assay, whilst the medium in the other was changed and the *Malassezia* extracts were re-applied accordingly. Before performing the assay, wells were washed and filled with 100 µl of fresh medium. The number of viable cells in each well was then detected using the

proliferation assay as described in section 5.3. On day 8, the assay was carried out again with the second plate in the same manner.

Results

Higher numbers of cells were detected in most of the wells after a 7 day incubation (with or without *Malassezia* extracts), indicating that keratinocytes cultured with *Malassezia* extracts were capable of proliferating (Figure 5.3 A-B). However, far fewer cells were detected when *Malassezia* extracts were used at a concentration of 5 mg/ml compared to the others on day 4 (Figure 5.3 A), and only a few viable cells were detected on day 8 (Figure 5.3 B), indicating that *Malassezia* extracts at the concentration of 5 mg/ml inhibited cell growth and led to cell death. This concentration was therefore abandoned in the subsequent experiments due to its cytotoxic effect on canine keratinocytes. Although the difference in number between cells grown without and with *Malassezia* extracts at concentrations of 5×10^{-1} - 5×10^{-5} was not dramatic in this experiment, repetition of similar experiments was required in order to obtain replicates for statistical analyses.

The results obtained on day 4 generally fell in the middle of the standard curve (containing two fold dilutions from 2×10^5 to 3125 cells per well), whereas those on day 8 were approaching the upper limit. Furthermore, the majority of the wells were confluent on day 8 when observed microscopically. Since a cessation of cell growth can be expected with confluent cultures, and detecting cells in their proliferation phase was considered to be ideal, the proliferation assay was performed on day 4 in subsequent experiments.

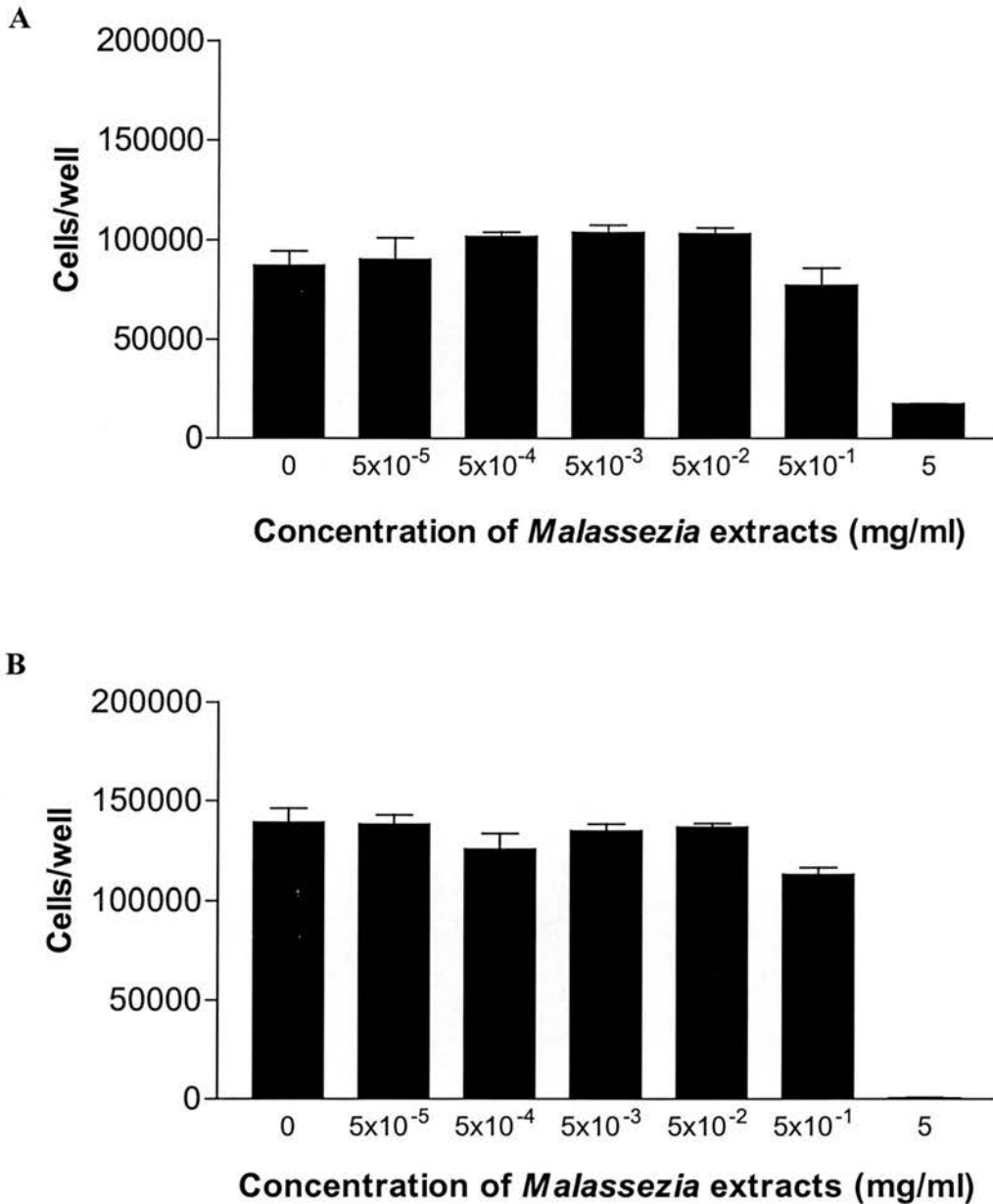


Figure 5.3 A-B Evaluation of different concentrations of *Malassezia* extracts and culture periods for culture with canine keratinocytes

A: Results obtained on day 4. B: Results obtained on day 8. Each bar represents the mean and standard error of the mean of quadruplicates. Far fewer cells were detected in the group cultured with *Malassezia* extracts at a concentration of 5 mg/ml on both days, indicating that high concentrations may have cytotoxic effects on keratinocytes. Although more cells were seen on day 8 than day 4, indicating that the cells were able to proliferate, confluent cultures were observed on day 8 indicating a decrease in activity. Day 4 was therefore deemed to be the optimal time to perform the cell proliferation assay.

5.4.2 Effects of *Malassezia* extracts on keratinocyte proliferation

Aims

In this section, the protocol and extract concentrations determined in section 5.4.1 were used to further investigate the effects of *Malassezia* extracts on keratinocyte proliferation. In order to examine the role of proteases in keratinocyte proliferation as well as other proteins in the *Malassezia* extracts, an extraction buffer made with and without protease inhibitors was used in this experiment.

Methods

Keratinocytes harvested from tissue culture flasks were washed, counted in a haemocytometer and seeded into 96-well plates at 2×10^4 cells/cm² in complete medium as well as into flasks to be used for generation of a standard curve in the cell proliferation assay. After 24 hours, the medium was changed in order to remove non-adherent cells. Fresh medium and *Malassezia* extracts made with and without protease inhibitors were added into wells at a final concentration of 5×10^{-1} - 5×10^{-5} mg/ml (see sections 2.5.2 and 2.7 for *Malassezia* extract preparation and sterilisation). Wells containing medium alone and PBS were used as controls. All wells were plated in at least quadruplicate for each experiment, and the plates were incubated for 3 days. Wells were washed and filled with 100 μ l of fresh medium on the day of the assay and the number of viable cells in each well was measured using the colourimetric cell proliferation assay described earlier (section 5.3). The experiment was repeated five times and the results were combined to give more replicates for each group in the statistical analyses. One-way ANOVA (Prism[®], Graphpad Inc., San Diego, USA) was used to compare the number of keratinocytes cultured with or without various concentrations of *Malassezia* extracts, and

significance was set at $P < 0.05$.

Results

The results obtained from 5 separate experiments are shown in Figure 5.4 A-B. There was no significant difference in cell numbers between any group of keratinocytes cultured with 5×10^{-1} - 5×10^{-5} mg/ml of extracts, either with or without protease inhibitors, or PBS which was the negative control ($P > 0.05$).

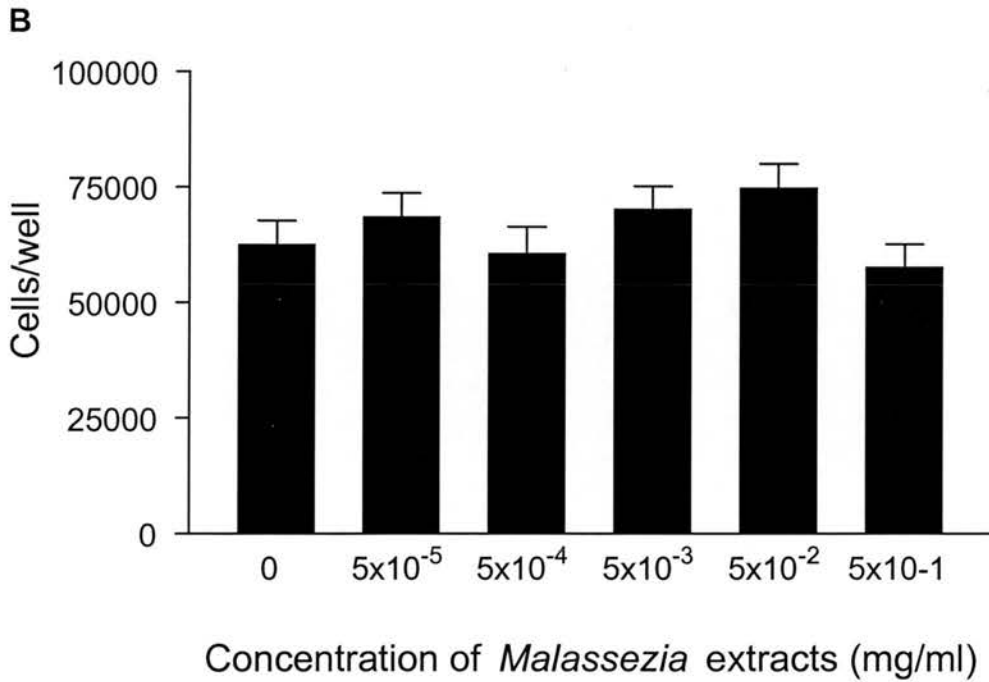
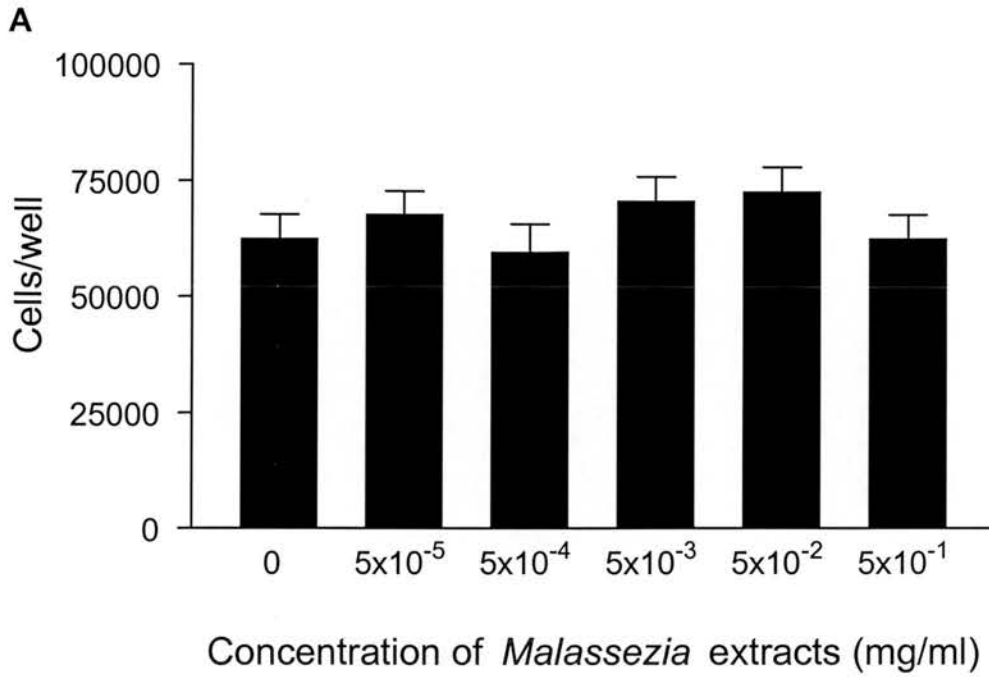


Figure 5.4 A-B Dose response of canine keratinocytes to *Malassezia* extracts

A: Extracts produced using buffer containing protease inhibitors. B: Extracts without protease inhibitors. Data shown are the mean of five separate experiments and error bars show the standard error of the mean. The differences between groups were analysed using one-way ANOVA and no significant difference was found between groups.

5.5 Culture of keratinocytes with culture supernatants from *M. pachydermatis*

5.5.1 Establishment of assay conditions for culture of keratinocytes with culture supernatants from *M. pachydermatis*

Aims

Having determined the optimal conditions to perform the proliferation assay on keratinocytes cultured with *Malassezia* extracts, the same conditions were evaluated for their use on cells grown with culture supernatants from *M. pachydermatis*. Also, different dilutions of the supernatants were assessed for their cytotoxic effects on canine keratinocytes.

Methods

Keratinocytes cultured in flasks were harvested, counted in a haemocytometer and plated into a 96-well plate at the density of 2×10^4 cells/cm² (100 μ l/well). Some cells were also seeded into flasks to be used for generating a standard curve in the cell proliferation assay. Non-adherent cells were removed the next day and the wells were filled with 100 μ l of fresh medium. An aliquot of the concentrated *Malassezia* culture supernatants that had been sterilized (see sections 2.6 and 2.7 for preparation and sterilisation) was diluted 10-fold with PBS and dilutions of 1/10 – 1/1,000 as well as undiluted supernatants were added into wells in quadruplicate. Due to the high protein content in the Sabouraud liquid medium, it was not possible to quantify the amount of specific *Malassezia* proteins in the supernatants. Their concentrations were therefore expressed as dilution ratios. A volume of 100 μ l of undiluted sterile Sabouraud liquid medium or PBS was used in control wells. The plate was incubated at 37°C with 5 % CO₂ in air for 3 days and the cell proliferation assay was performed

as described earlier.

Results

After culturing keratinocytes with *Malassezia* culture supernatants for 3 days, the number of viable cells detected in most of the groups was within the range of the standard curve generated from two-fold dilutions from 2×10^5 to 3125 cells per well (Figure 5.5), validating the use of the protocol described above. Nevertheless, undiluted *Malassezia* culture supernatants and sterile Sabouraud liquid medium suppressed the growth of keratinocytes and caused cell death. In contrast, this cytotoxic effect was not seen in the groups cultured with *Malassezia* culture supernatants diluted 1/10 – 1/1,000 and PBS. These dilutions were therefore used in subsequent experiments.

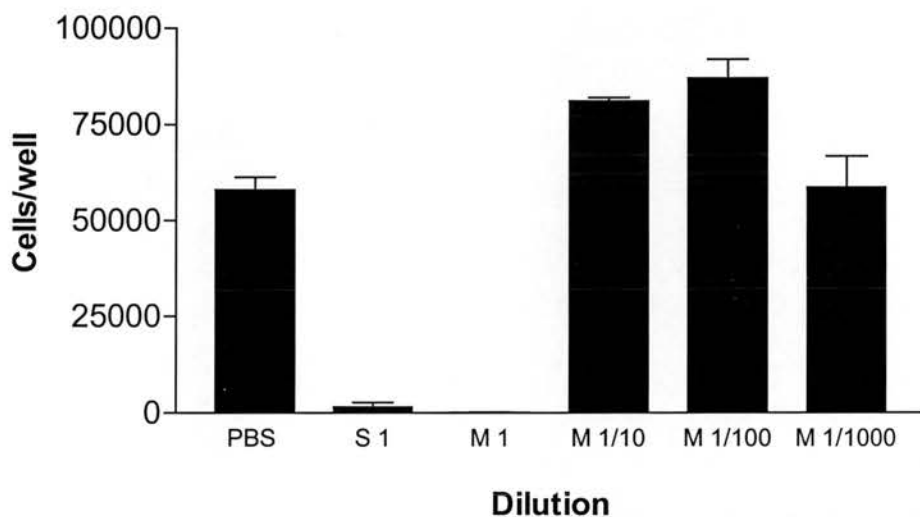


Figure 5.5 Evaluation of various dilutions of culture supernatants from *M. pachydermatis* for culture with canine keratinocytes

Undiluted *Malassezia* culture supernatants and three dilutions were evaluated. After a 3 day incubation, the groups cultured with undiluted *Malassezia* culture supernatants and sterile Sabouraud liquid medium showed very few viable cells, indicating that this concentration had cytotoxic effect on keratinocytes, and was therefore not suitable for use in co-culture. Each bar represents the mean and standard error of the mean of quadruplicates. S: sterile Sabouraud liquid medium; M: culture supernatants from *M. pachydermatis*.

5.5.2 Effects of culture supernatants from *M. pachydermatis* on keratinocyte proliferation

Aims

The aim of this experiment was to investigate the effects of culture supernatants from *M. pachydermatis* on the proliferation of canine keratinocytes by measuring the number of viable cells after culturing keratinocytes with various dilutions of *Malassezia* culture supernatants using the protocol and dilutions assessed in section 5.5.1.

Methods

Keratinocytes were plated into 96-well plates at 2×10^4 cells/cm² in complete medium. For generating a standard curve in the cell proliferation assay, some cells were also seeded into flasks. The medium was changed the next day to remove non-adherent cells. Concentrated *Malassezia* culture supernatants and sterile Sabouraud liquid medium were diluted 10-fold with PBS and dilutions of 1/10 – 1/10,000 were added into wells in at least quadruplicate for each experiment. The plates were incubated at 37°C with 5 % CO₂ in air for 3 days and the wells were washed on the day of the assay before adding 100 µl of fresh medium. The cell proliferation assay was performed as described earlier and the experiment was repeated on four different occasions to obtain more replicates for statistical analyses. Results obtained from repeated experiments were combined and T-tests (Prism®, Graphpad Inc., San Diego, USA) were used to compare the cell numbers of keratinocytes incubated with culture supernatants of *M. pachydermatis* and sterile medium for each dilution ratio. Significance was set at $P < 0.05$.

Results

Figure 5.6 shows the combined results of four repeated experiments. No significant difference was found between each group of cells incubated with culture supernatants from *M. pachydermatis* at the four dilutions compared to the individual negative controls ($P > 0.05$).

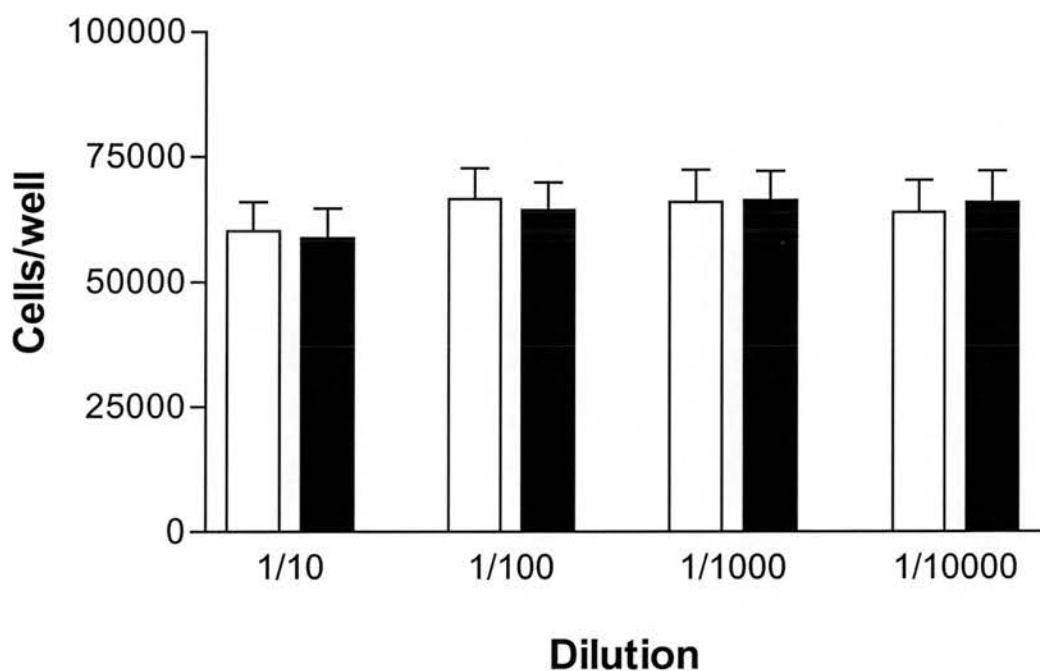


Figure 5.6 Dose response of canine keratinocytes to culture supernatants from *Malassezia pachydermatis*

Data shown are the mean of four experiments and error bars show the standard error of the mean. The differences between cells cultured with supernatants and sterile medium were analysed using t-tests. There was no significant difference between the two groups at any dilution. White bars: sterile medium, Black bars: culture supernatants from *Malassezia*.

5.6 Discussion

5.6.1 Validation and optimization of the cell proliferation assay

The initial aim of the experiments in this chapter was to validate the use of a colourimetric proliferation assay with canine keratinocytes. Proliferation assays provide several advantages over traditional manual counting and have been widely used in proliferation and cytotoxicity studies. The tetrazolium assay, first described by Mosmann (1983) and generally known as the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, is a colourimetric assay based on the bio-reduction of a tetrazolium salt to a coloured substance, formazan. The procedure involves a solubilisation step to dissolve the formazan product of MTT, which is a crystalline precipitate, prior to reading absorbance. This can be troublesome and the difficulty associated with incomplete solubilisation of the crystals is the main drawback of this assay. The second generation assays use tetrazolium salts that form soluble formazans, such as MTS (3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and XTT (sodium (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide), and an electron coupling reagent, phenazine methosulfate (PMS). However, the MTS/PMS mixture has been demonstrated to be much more stable than XTT/PMS (Goodwin *et al.* 1995), and the MTS assay has been shown to be equivalent to the ³H-thymidine incorporation assay using human epidermal keratinocytes (Kaplan *et al.* 1995). The reagent used in this study is an improvement over the original MTS assay and contains stable pre-combined MTS/PMS. Using this reagent, a linear correlation between the absorbance and cell number per well was demonstrated with logarithmical scales in section 5.2, indicating its potential value for application in canine keratinocyte research requiring rapid and quantitative measurement.

In order to further validate the use of this proliferation assay on canine keratinocytes and to verify that the culture conditions for keratinocytes were optimal, the assay was used to compare the proliferative activity of canine keratinocytes cultured with various combinations of growth factors (section 5.3). The results showed that canine keratinocytes had a higher proliferation rate in medium containing both cholera toxin (CT) and EGF, as was shown by Wilkinson *et al.* (1987) using manual counting with a haemocytometer. Both CT and EGF act directly to increase the growth and life span of cultured keratinocytes (Wilkinson *et al.* 1987). CT is an 84 kDa protein composed of two subunits, A and B (Okada *et al.* 1982), and it stimulates the growth of keratinocytes via increasing intracellular cyclic adenosine 5'-monophosphate (cAMP) (Green 1978; Okada *et al.* 1982). The effect of CT on keratinocyte proliferation was shown to be biphasic, with a stimulatory effect at low cell densities and inhibitory effects after the culture reached confluence (Okada *et al.* 1982). In contrast, EGF increased keratinocyte proliferation especially at high cell densities, and it also facilitated successful cell cultivation as well as colony formation (Rheinwald & Green 1977). It has been proposed that EGFs induce keratinocyte proliferation primarily by enhancing cell migration to enlarge colonies (Barrandon & Green 1987). By adding CT and EGF to culture medium, they enhance colony formation and cell growth at both low and high cell densities.

5.6.2 Effects of extracts from *M. pachydermatis* on keratinocyte proliferation

The main purpose of this study was to determine if extracts and culture supernatants from *Malassezia pachydermatis* could directly induce proliferation of canine keratinocytes. This was suggested by a preliminary study using live organisms (von Tschärner *et al.* 1999). The proteins and proteases extracted from *M. pachydermatis*

or secreted by the organisms might have a direct influence on the proliferation of keratinocytes via their binding to receptors on the surface of keratinocytes triggering a cascade of proliferative signals. They might also promote the production of stimulatory factors for keratinocyte proliferation such as EGF, FGF, IGF-1, IL-1, IL-6 and GM-CSF, which could form an autocrine and paracrine regulatory network resulting in activation of the secretory keratinocyte and its neighboring cells. The findings of this study, however, showed that *Malassezia* extracts did not affect keratinocyte proliferation, either with extracts produced with or without protease inhibitors. These results suggest that products from *M. pachydermatis* do not directly lead to the epidermal hyperplasia seen in *Malassezia* dermatitis.

Similarly, the proliferation of keratinocytes was not affected by co-culturing keratinocytes with culture supernatants from *Malassezia pachydermatis*. The protein profile shown in Figure 2.6 indicates that proteins with molecular weights between 66 and 45 kDa were generated in the liquid medium or released from *Malassezia* organisms during culture. It is unclear what these products are without further purification and analysis. However, protease activity has been demonstrated in yeast-free supernatants when *M. pachydermatis* was cultured in liquid medium (Mathieson *et al.* 1998). It is likely, therefore, that the culture supernatants used in this study contained proteases as well. Enzyme secretion is one of the virulence factors possessed by many microorganisms. Protease is an important virulence factor of candidiasis in humans (Macdonald & Odds 1983; Cassone *et al.* 1987), and a keratinolytic protease of *Candida albicans* has been shown to digest human stratum corneum *in vitro* (Negi *et al.* 1984). Enzymes produced by *Malassezia* organisms are generally considered to be potential pathogenic factors. Coutinho and Paula (2000)

demonstrated that all the strains of *Malassezia pachydermatis* isolated from dogs with otitis and dermatitis showed protease activity. Protease released by *M. pachydermatis* was proposed to be the mediator of itch at free nerve endings in the skin and contribute to the prominent pruritus seen in affected dogs (Mason & Evans 1991). However, little work has been done looking at the role of proteases in the pathogenesis of histopathological changes associated with *Malassezia* dermatitis. They might promote mitosis of keratinocytes as with the stimulation of keratinocytes by neutrophil proteases (Rogalski *et al.* 2002). However, in this study, there was no difference in the growth rate of keratinocytes cultured with *Malassezia* extracts produced either with or without protease inhibitors, or keratinocytes cultured with supernatants from *M. pachydermatis* compared to sterile medium. These results indicate that proteases or other proteins secreted by the organism do not cause changes in keratinocyte proliferation *in vitro*. Taken together, the results of this chapter suggest that neither of the mechanisms mentioned above happened *in vitro*. However, further studies are required to determine if extracts and culture supernatants from *M. pachydermatis* could alter cytokine expression in cultured canine keratinocytes. If *M. pachydermatis* can induce keratinocyte proliferation directly, it is likely that cell to cell interaction is required between the whole organism and the keratinocyte. This hypothesis is tested in the next chapter.

This study was performed using healthy dog skin. It is important to consider the possibility that there may be a difference in proliferation rate in response to *M. pachydermatis* between normal and *Malassezia*-infected skin. Higher thymidine incorporation has been demonstrated in basal cells of *Candida*-infected skin compared to normal skin of guinea pigs (Sohnle & Kirkpatrick 1978). Whether this

is the case in dogs with *Malassezia* dermatitis remains to be investigated. It is also possible that skin from dogs with atopic dermatitis might exhibit different proliferative responses to those of normal dogs, although this awaits further study.

In summary, the results of this chapter have shown that *Malassezia* extracts, either with or without protease inhibitors, do not cause proliferation of normal canine keratinocytes *in vitro*. Likewise, culture supernatants from *Malassezia pachydermatis* had no effect on the growth rate of keratinocytes. It seems likely, therefore, that the excessive epidermal proliferation associated with *Malassezia* dermatitis is induced by other mechanisms.

Chapter 6

THE EFFECT OF CO-CULTURE OF CANINE KERATINOCYTES WITH *MALASSEZIA* *PACHYDERMATIS* ORGANISMS

6.1 Introduction

The studies described in chapter 5 indicated that extracts or culture supernatants from *Malassezia pachydermatis* had no stimulatory effect on the proliferation of canine keratinocytes, regardless of whether or not they were prepared with protease inhibitors. In this chapter, the influence of the interaction between viable *Malassezia* organisms and canine keratinocytes was investigated.

It is possible that direct contact between an organism and keratinocytes could be required to stimulate a proliferative response. This response would initially depend on adherence of the organism to the cell surface, followed by activation of signal transduction mechanisms and culminating in modulation of the cell cycle or up-regulation of growth factor secretion. Adherence is generally considered to play an important role in the colonisation and infection of many microorganisms. The initial stage in skin infections likely involves interaction between the organism and keratinocytes, and the adhesion molecules that mediate this process have been widely investigated in pathogenic bacteria and fungi. Multiple surface proteins of *Staphylococcus aureus* have been reported to play a role in adhesion to human keratinocytes *in vitro*, including staphylococcal protein A, clumping factor, coagulase, and fibronectin-binding proteins A and B (Mempel *et al.* 1998). A specific surface-exposed protein, M protein, has been found to be an adhesin of *Streptococcus*

pyogenes that allows adherence to keratinocytes (Okada *et al.* 1994). A surface protein of keratinocytes, membrane cofactor protein (MCP or CD46), has been demonstrated to bind to a region of M protein, known as the C repeat domain, and thus serve as the receptor for adherence of *S. pyogenes* (Okada *et al.* 1995; Perez-Casal *et al.* 1995). Wang *et al.* (1997) studied the response of keratinocytes to adherent and non-adherent *S. pyogenes* and revealed distinct patterns of keratinocyte reaction induced by the two different strains. These included cytokine gene expression, prostaglandin production and membrane damage, suggesting that adhesin expression of *S. pyogenes* and its interaction with keratinocytes modulated keratinocyte responses during skin infection. In dogs, the adhesion molecules that mediate the adherence of *Staphylococcus intermedius* to canine keratinocytes and their interactions have not been documented (McEwan 2000; Saijonmaa-Koulumies & Lloyd 2002; Forsythe *et al.* 2002).

The interaction between adhesins of *Candida albicans* and host cell ligands has been the subject of numerous studies due to the importance of this yeast as a pathogen in humans. The adhesins and ligands involved in the adherence of *C. albicans* to epithelial cells include lectin-like mannoprotein, mannan factor 6 and chitin possessed by the yeast, and glucoside ligands with terminal fucosyl or *N*-acetylglucosamine residues present on the host cell surface (reviewed by Kennedy 1988; Calderone *et al.* 1994; Fukazawa & Kagaya 1997). In contrast, the adherence of *Malassezia* spp. has not been studied extensively. It was found that *Malassezia furfur* adhered to human stratum corneum cells *in vitro* in a dose, time and temperature-dependent manner, and the adherence to cells from different anatomical sites did not differ significantly (Faergemann *et al.* 1983). One study looking at the

effect of *Malassezia furfur* on an adhesion molecule of human keratinocytes reported that there was no difference in the expression of mRNA for intercellular adhesion molecule 1 (ICAM 1) between control and *Malassezia furfur*-treated keratinocyte cell lines, thus suggesting that *Malassezia furfur* did not modulate ICAM 1 expression (Baroni *et al.* 2001a). In accordance with the study on *Malassezia furfur*, *Malassezia pachydermatis* was found to adhere to canine corneocytes *in vitro* in a dose and time-dependent manner (Bond & Lloyd 1996b). Furthermore, trypsin-sensitive proteins or glycoproteins on the yeast cell wall and on the corneocyte surface were shown to be important in the adherence of *M. pachydermatis in vitro* (Bond & Lloyd 1996b). Carbohydrate adhesins and/or ligands were also involved in adherence of *M. pachydermatis in vitro*, however, apparent variability has been shown between strains (Bond & Lloyd 1998). More recently, the adherence of *M. pachydermatis* to cornified epithelial cells from canine ear canals was suggested to be mediated by lipid (Masuda *et al.* 2001). It seems likely, therefore, that the candidate adhesins and ligands for *M. pachydermatis* include proteins, carbohydrates and lipids. However, the precise identities of these molecules are not yet known. The interaction between adhesins of *M. pachydermatis* and ligands on the keratinocyte surface could trigger intracellular signal transduction and thus modulate the functions of keratinocytes, such as proliferation. Very little research in this respect has been reported.

The main aim in this chapter was to further investigate the role of *Malassezia pachydermatis* in canine keratinocyte proliferation by evaluating the growth of keratinocytes co-cultured with live *Malassezia* organisms by cell counting and immunohistochemistry using antibodies against proliferation markers. The effect of *Malassezia* organisms on the viability of canine keratinocytes was also investigated

using reagents to detect apoptotic cells in keratinocyte monolayers co-cultured with *M. pachydermatis*. The first part of this chapter describes the validation of cell counting methods and keratinocyte quantification. The immunohistochemistry staining is reported in the second part.

EFFECTS OF *MALASSEZIA PACHYDERMATIS* ON THE PROLIFERATION OF CANINE KERATINOCYTES

6.2 Evaluation of the Celltiter 96[®] colourimetric assay to enumerate canine keratinocytes co-cultured with *M. pachydermatis* organisms

6.2.1 Assessment of reactivity between assay reagent and *Malassezia* organisms

Aims

In chapter 5, the Celltiter 96[®] colourimetric cell proliferation assay was used to determine keratinocyte numbers when cultured with extracts and culture supernatants from *Malassezia pachydermatis*. The initial aim of this part of the study was to test the feasibility of using this assay to count keratinocytes co-cultured with live *Malassezia* organisms, and to test whether or not the yeast cells reacted with the assay reagent and generated a signal.

Methods

Keratinocytes grown in tissue culture flasks were harvested by trypsinisation (section 2.19), washed, and counted in a haemocytometer (section 2.17). They were then plated into a 96-well plate (100 µl/well, 2×10^4 cells/cm²) and cultured in complete medium at 37°C in a humidified atmosphere with 5 % CO₂ in air. Some cells were also plated in flasks to be used for generating a standard curve in the cell proliferation assay. After 24 hours, the medium in the plate was replaced with complete WME without Penicillin, Streptomycin, and Fungizone (PSF). On day 3, the medium was changed again to remove any trace of PSF and the plate was incubated at 37°C while preparing the *Malassezia* suspension.

A strain of *Malassezia pachydermatis* originating from the ear canal of a dog with

Malassezia otitis was cultured on Sabouraud Dextrose agar for 48 hours at 37°C before harvesting (section 2.4). *Malassezia* colonies were carefully harvested and suspended in PBS for a washing procedure that consisted of three cycles of centrifugation at 500 g for 5 minutes followed by removal of the supernatant and resuspension in PBS. After the last washing cycle, the cells were resuspended in complete WME without PSF, counted in a haemocytometer, and adjusted to a concentration of 10^7 yeast cells/ml. The suspension was then serially diluted ten-fold (10^7 - 10^3) and 100 µl of each dilution was added in quadruplicate into both empty wells and wells containing 100 µl of medium and growing canine keratinocytes, resulting in a density of 10^6 - 10^2 yeast cells per well. Another 100 µl of complete WME without PSF was added into wells with yeast cells alone, so that all wells contained 200 µl of medium. Wells with keratinocytes alone or 200 µl of medium served as controls. The plate was incubated at 37°C with 5 % CO₂ in air for a further 24 hours, and the cell proliferation assay was performed in the same manner as described in the previous chapter. Briefly, keratinocytes grown in flasks were trypsinised, washed and counted in a haemocytometer for generating a standard curve. A volume of 200 µl of cell suspension (containing two fold dilutions from 1×10^5 to 3125 cells per well) was added in triplicate to empty wells. Wells used for background controls were filled with 200 µl of medium alone. Forty microliters of Celltiter 96® AQueous One Solution reagent (Promega, UK) was then added to all used wells and the plate was incubated for a further 3 hours. The absorbance of the reaction was evaluated using an ELISA reader at a wavelength of 490 nm and a reference wavelength of 630 nm (section 2.25). The absorbance of wells containing keratinocytes and various numbers of *Malassezia* organisms as well as yeast cells alone was compared with the standard curve and the results were calculated as cells

per well, all of which were generated automatically by the ELISA reader. The signal generated by *Malassezia* cells alone was expressed as the equivalent number of keratinocytes that generated the same amount of colour change.

Results

The results of the different groups (keratinocytes alone, *Malassezia* organisms alone, keratinocytes plus *Malassezia* organisms) are shown in Figure 6.1 A. *Malassezia* organisms were shown to react with the assay reagent and generate detectable signals in a dose response manner. Stronger signals were seen in the wells containing higher numbers of yeast cells. This indicated that the signals detected in wells containing both keratinocytes and *Malassezia* organisms were generated not only from the keratinocytes but also the yeast cells, and this was especially so in keratinocytes cultured with the highest density of *Malassezia*. Therefore, the signals detected from wells containing yeast cells alone were treated as background controls, and subtracted from those obtained in wells containing both keratinocytes and *Malassezia* organisms (Figure 6.1 B). After subtraction, the results showed that more keratinocytes were present in the groups co-cultured with higher numbers of *Malassezia* cells, suggesting that *Malassezia* organisms stimulated keratinocyte proliferation. However, this was not in agreement with microscopic observation of the monolayers. Fewer keratinocytes were observed at the bottom of wells containing higher numbers of *Malassezia* cells compared to control wells where keratinocytes were cultured alone, and this was particularly apparent in those with 10^6 yeast cells (Figure 6.2 A-B). A possible explanation for this discrepancy might be that there were detached keratinocytes floating in the medium, the number of which was difficult to determine by microscopic observation. These cells could be viable or

dead, but could still possibly be detected by the assay reagent. In order to test whether the reagent could detect dead cells, the proliferation assay was performed with dead keratinocytes in the following experiment.

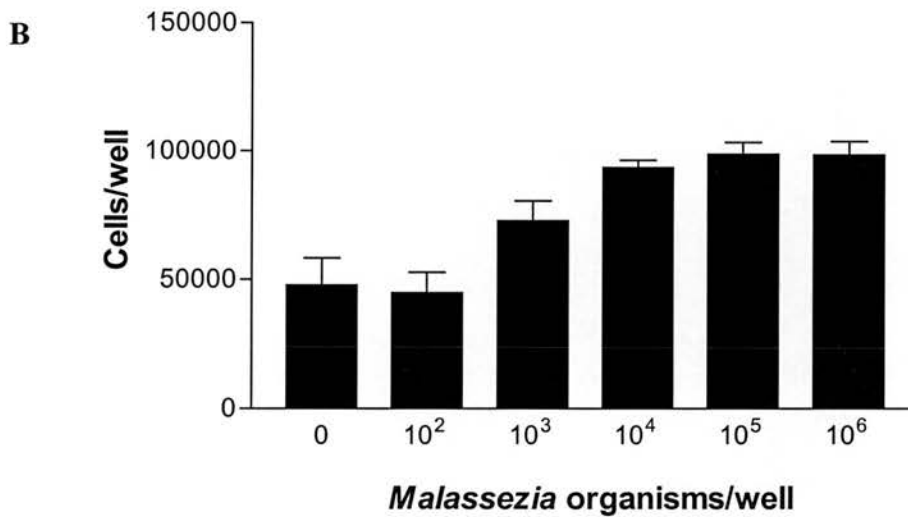
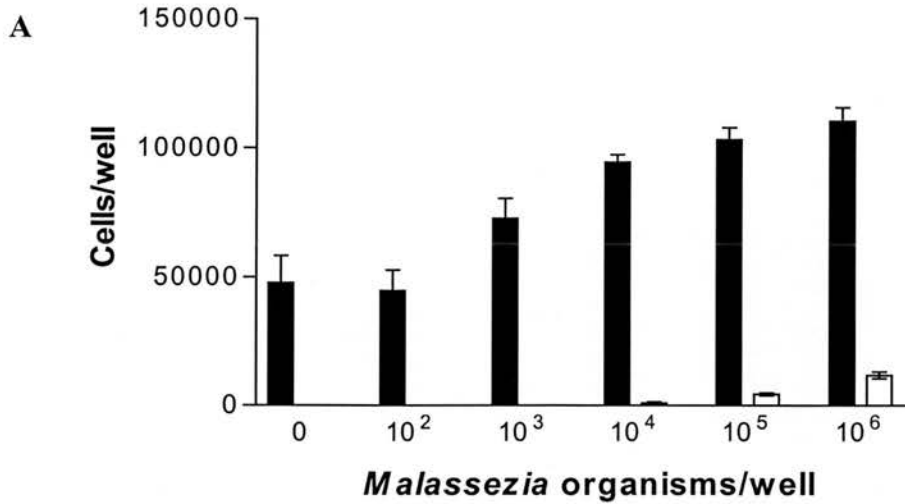


Figure 6.1 A-B Use of the Celltiter 96® assay to quantify keratinocyte numbers in co-culture experiments

A: Comparison of the results obtained from keratinocytes co-cultured with various densities of *Malassezia* organisms (Black bars) to those from yeast cells alone (White bars). Each bar represents the mean and standard error of the mean of quadruplicates. The signals generated by *Malassezia* organisms were expressed as the equivalent number of keratinocytes that generated the same amount of colour change, and the results showed a dose response trend, indicating that *M. pachydermatis* reacted with the assay reagent. Therefore, the yeast cells also contributed to the colour changes detected in wells containing both keratinocytes and *Malassezia* organisms.

B: Results obtained after subtracting the numbers generated by *Malassezia* organisms alone from those by keratinocytes and yeast cells. More keratinocytes were shown to be present in the groups with higher numbers of *Malassezia* organisms. However, this was not in agreement with microscopic observation of the monolayers (see text for details).

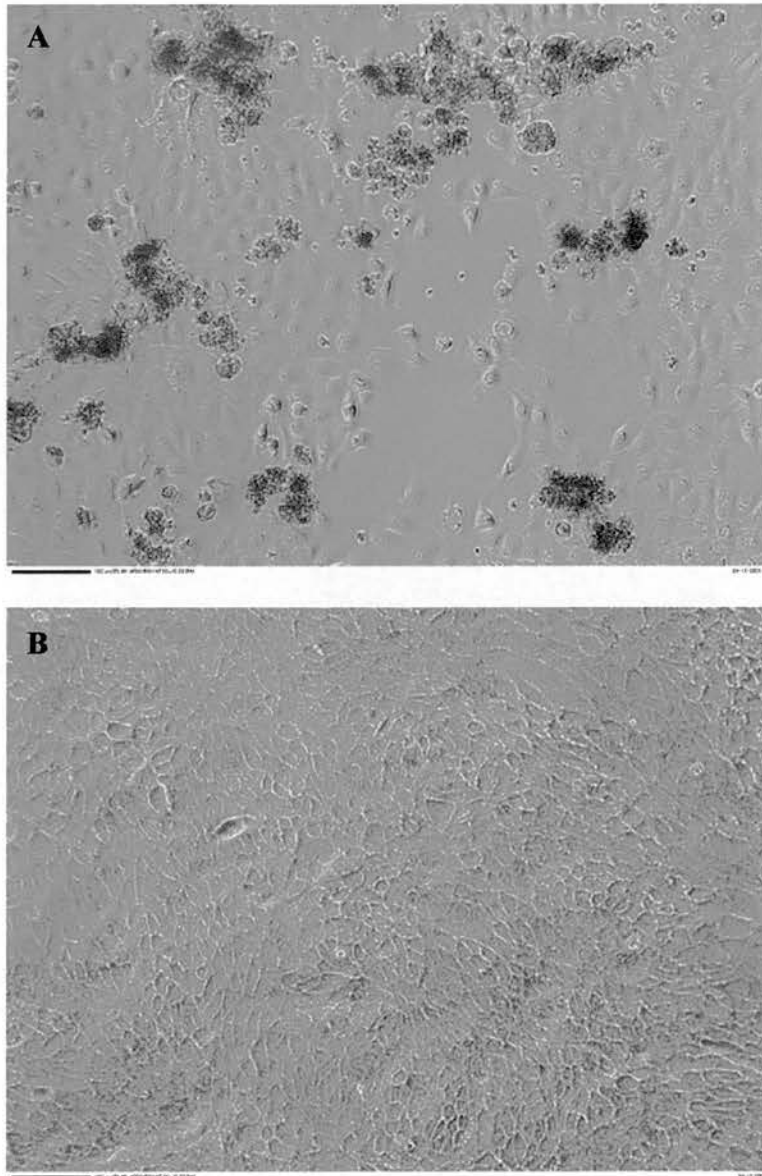


Figure 6.2 A-B Comparison of the morphology of canine keratinocyte cultures co-cultured with or without *Malassezia pachydermatis* organisms (x100)

A: Keratinocytes co-cultured with 10^6 *Malassezia* organisms. B: Keratinocytes cultured alone. Apparent difference in the degree of confluence was observed between the two cultures.

Scale bars = 100 μm.

6.2.2 Confirmation of reagent specificity for viable cells

Aims

Discrepancies were found between the results obtained using the cell proliferation assay and those observed by microscopic examination as mentioned in the previous section. The aim of this experiment was to test the hypothesis that the assay reagent detected dead keratinocytes in the medium which caused the inconsistency. In order to assess whether or not the reagent reacted with dead cells, the assay was carried out using keratinocytes that had been killed by subjecting them to a freeze/thaw cycle.

Methods

Keratinocytes cultured in flasks were harvested, counted in a haemocytometer and plated into a 96-well plate at a density of 2×10^4 cells/cm² (100 µl/well) in complete medium. Non-adherent cells were removed the next day and the wells were filled with 100 µl of complete WME without PSF. After a further one day incubation, the medium was changed again and the plate was frozen at -80°C for an hour followed by thawing at 37°C. The *Malassezia* suspension was prepared as described in the previous section and 10^6 - 10^2 yeast cells per well were added in quadruplicate to empty wells and wells containing keratinocytes. Control wells contained keratinocytes alone or 200 µl of medium. The plate was incubated at 37°C with 5 % CO₂ in air for a further 24 hours and the cell proliferation assay was performed as described earlier.

Results

The results obtained from the groups with or without keratinocytes are shown in Figure 6.3 A. There was a clear difference in cell numbers in the groups containing

keratinocytes and *Malassezia* organisms when compared to those in Figure 6.1 A. In this experiment, the cell numbers detected in wells with or without keratinocytes were very similar at every density of *Malassezia* organisms used, and after the numbers obtained from *Malassezia* cells alone were subtracted from those generated in wells with both keratinocytes and yeast cells, the figures were close to zero (Figure 6.3 B). These results suggested that the signals detected in wells containing keratinocytes and *Malassezia* organisms were generated predominantly from the yeast cells, but not from the keratinocytes that had been killed by a cycle of freezing and thawing, indicating that the assay reagent did not react with dead cells. This could not, therefore, be used to explain the discordant results in section 6.2.1. Although it was likely that there were some viable cells in the medium which were detected by the reagent, the number of these cells was unlikely to be high enough to generate signals of the magnitude that were detected in Figure 6.1, since unattached keratinocytes are unable to proliferate (Gniadecki 1998). Due to the difficulties in explaining the discrepancy between the results obtained using the cell proliferation assay and the observations of cell numbers by microscopy, this assay method was deemed to be unsuitable for counting canine keratinocytes co-cultured with *Malassezia* organisms.

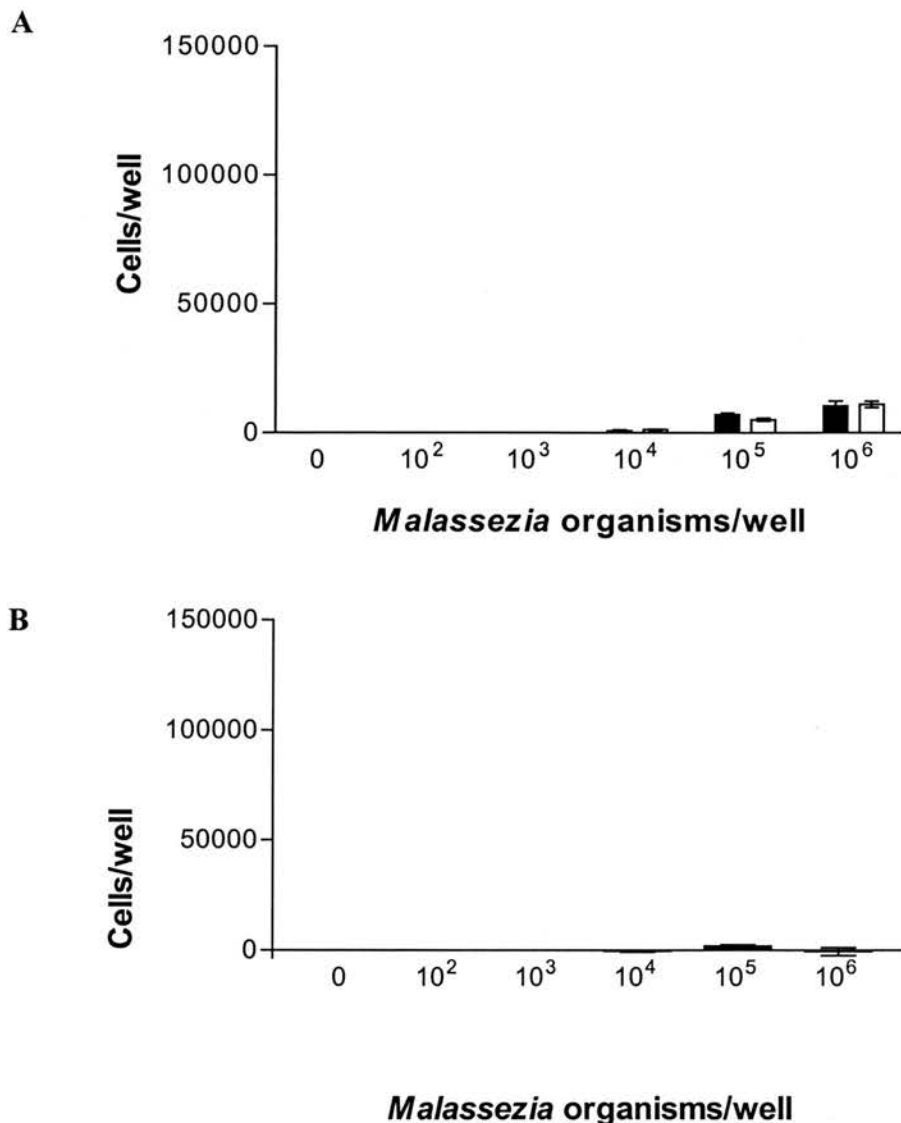


Figure 6.3 A-B Assessment of the reactivity of Celltiter 96[®] assay reagent with keratinocytes subjected to a cycle of freezing and thawing

A: Comparison of the results obtained from dead keratinocytes cultured with various densities of *Malassezia* organisms to those from yeast cells alone. Each bar represents the mean and standard error of the mean of quadruplicates. The numbers shown by dead keratinocytes were very similar to those by *Malassezia* organisms alone, regardless of the density of yeast cells they were co-cultured with, suggesting that the assay reagent did not react with dead keratinocytes and the colour changes were predominantly generated by *Malassezia* organisms. Black bars: keratinocytes cultured with *Malassezia* organisms, Open bars: *Malassezia* organisms alone.

B: Subtraction of the numbers generated by *Malassezia* organisms alone from those by dead keratinocytes and yeast cells. The results further confirmed the similarity of cell numbers detected in the two groups at any density of *M. pachydermatis*.

6.3 Investigation into the effect of *Malassezia pachydermatis* on cultured canine keratinocytes using manual counting

Aims

Due to suspected technical problems when using the Celltiter 96® proliferation assay to quantify keratinocytes in co-culture experiments, an alternative method of quantification was evaluated. This involved direct manual cell counting using a haemocytometer. Although this technique would be more laborious, it would allow counting of the keratinocyte population in both the monolayer and the medium. It would also allow easy discrimination between keratinocytes and yeast cells.

Methods

Keratinocytes harvested from tissue culture flasks were washed, resuspended in complete WME and counted in a haemocytometer. In order to obtain greater numbers of cells to facilitate manual counting, 12-well tissue culture plates (in which the area of each well was much larger than that of a 96-well plate) were used in this experiment. Cells were plated into three 12-well plates at a density of 1.5×10^4 cells/cm² (1 ml/well) and incubated at 37°C with 5 % CO₂ in air. After 24 hours, the medium was replaced with complete WME without PSF. On day 3, the medium was changed again to ensure no PSF remained and the keratinocytes were co-cultured with *Malassezia* organisms suspended in complete WME without PSF at similar densities to the experiments performed in 96-well plates. Due to the limited number of wells in a 12-well plate, four densities of *Malassezia* organisms were used: 10^7 , 10^5 , 10^3 , and 10^2 per well (1 ml/well), each of which were added in duplicate in every plate. Keratinocytes cultured in 2 ml of complete WME without PSF and yeast cells acted as controls. Over the following three days, the morphology of the culture was

observed and photographed, and manual counting with a haemocytometer was performed with one plate on each day. Before trypsinising the cells attached to the plate, the medium in each well was collected and the cells in the medium were also counted. The cell viability was assessed by trypan blue exclusion (section 2.17). This experiment was repeated three times to confirm the reproducibility of results. The cells used in three repeated experiments were obtained from two different dogs and were at their second, third and fourth passage when the experiments were conducted.

In order to investigate the significance of the relationships between the number of keratinocytes per well and both the density of *Malassezia* organisms and the duration of *Malassezia*-keratinocyte co-cultures, standard generalised linear models were used on the data obtained from the monolayers and the media. The numbers of keratinocytes were log transformed to normalise the data, and the software Splus 2000 (Insightful Corporation, Seattle, USA) was used to performed the analyses. The potential effect of three repeated experiments was also taken into account by putting replicates in the model as a factor at the beginning of the analyses. Two factors, logarithm of *Malassezia* densities and day of co-culture, were then added into the model sequentially. The suitability of the model was checked by considering the error structures which were found to be normal. In all cases, a *P* value < 0.05 was taken to indicate significance.

Results

The results obtained from three experiments were very similar and are shown in Figures 6.4.1-6.4.3. Figures 6.4.1 A, 6.4.2 A and 6.4.3 A show the number of viable keratinocytes in the monolayer after co-culture with or without 10^2 , 10^3 , 10^5 , and 10^7

Malassezia organisms for one, two and three days respectively. There was a significant difference in log cells/well between the three repeated experiments ($F_{2, 87}=191.13$, $P<0.0001$), which might be due to the different sources and passages of the cells used. Despite this, there was a significant decrease in log cells/well as the density of *Malassezia* organisms increased ($F_{1, 86}=74.32$, $P <0.0001$). The tendency for fewer keratinocytes to be present in the monolayers co-cultured with higher numbers of *Malassezia* organisms was in accordance with the microscopic observations in the experiment using the cell proliferation assay (section 6.2.1). Furthermore, as the co-culture was lengthened, there was a significant increase in the total of log cells/well in the monolayer ($F_{1, 85}=38.62$, $P <0.0001$). However, the increase in cell numbers was mainly contributed by control keratinocytes and those co-cultured with low numbers of *Malassezia* organisms. The cell counts obtained from the keratinocyte monolayers in three experiments during the three-day co-culture are summarised in Figure 6.4.4. Rapid increases in keratinocyte numbers were seen on the last day of co-culture in control keratinocytes and those co-cultured with low numbers of *Malassezia* organisms (Black bars). By contrast, cells cultured with 10^5 or 10^7 *Malassezia* organisms showed minimal growth, and the latter exhibited the lowest cell counts during the three-day co-culture. In contrast to the trend towards confluence and dense cell populations shown by control keratinocytes, no apparent increase in cell density was observed microscopically in keratinocytes co-cultured with 10^7 *Malassezia* cells (Figure 6.5 A-F). These cells also showed abnormal morphology on the last day of co-culture (Figure 6.5 E).

Figures 6.4.1 B, 6.4.2 B and 6.4.3 B show the number of dead (red lines) and viable keratinocytes (blue lines) in the medium after co-culture with or without 10^2 , 10^3 , 10^5 ,

and 10^7 *Malassezia* organisms for one, two and three days respectively. There were also significant differences in the data of dead cells ($F_{2, 87}=5.32$, $P = 0.0066$) and viable cells ($F_{2, 87}=9.77$, $P = 0.0002$) in the medium between three repeated experiments, which might be due to the same reason described above. In contrast to the results shown by adherent keratinocytes, the cell counts in the medium revealed an opposite effect of co-culture with *Malassezia* organisms. There was a significant increase in log cells/well of dead cells in the medium with increasing number of *Malassezia* organisms ($F_{1, 86}=145.29$, $P < 0.0001$). However, there was no significant change in viable cells in the medium as the number of *Malassezia* organisms was increased ($F_{1, 86}=2.72$, $P = 0.1031$). Nevertheless, the number of both dead and viable cells in the medium increased significantly the longer the co-cultures were maintained ($F_{1, 85}=60.51$, $P < 0.0001$; $F_{1, 85}=5.66$, $P = 0.0196$).

In summary, keratinocytes cultured with higher numbers of *Malassezia* organisms exhibited a significantly lower proliferation rate. In contrast, the number of dead cells in the medium rose significantly as the density of *Malassezia* organisms increased. Taken together, these results indicated that *Malassezia* organisms did not stimulate proliferation of keratinocytes. However, they induced these cells to detach from the monolayer and undergo cell death.

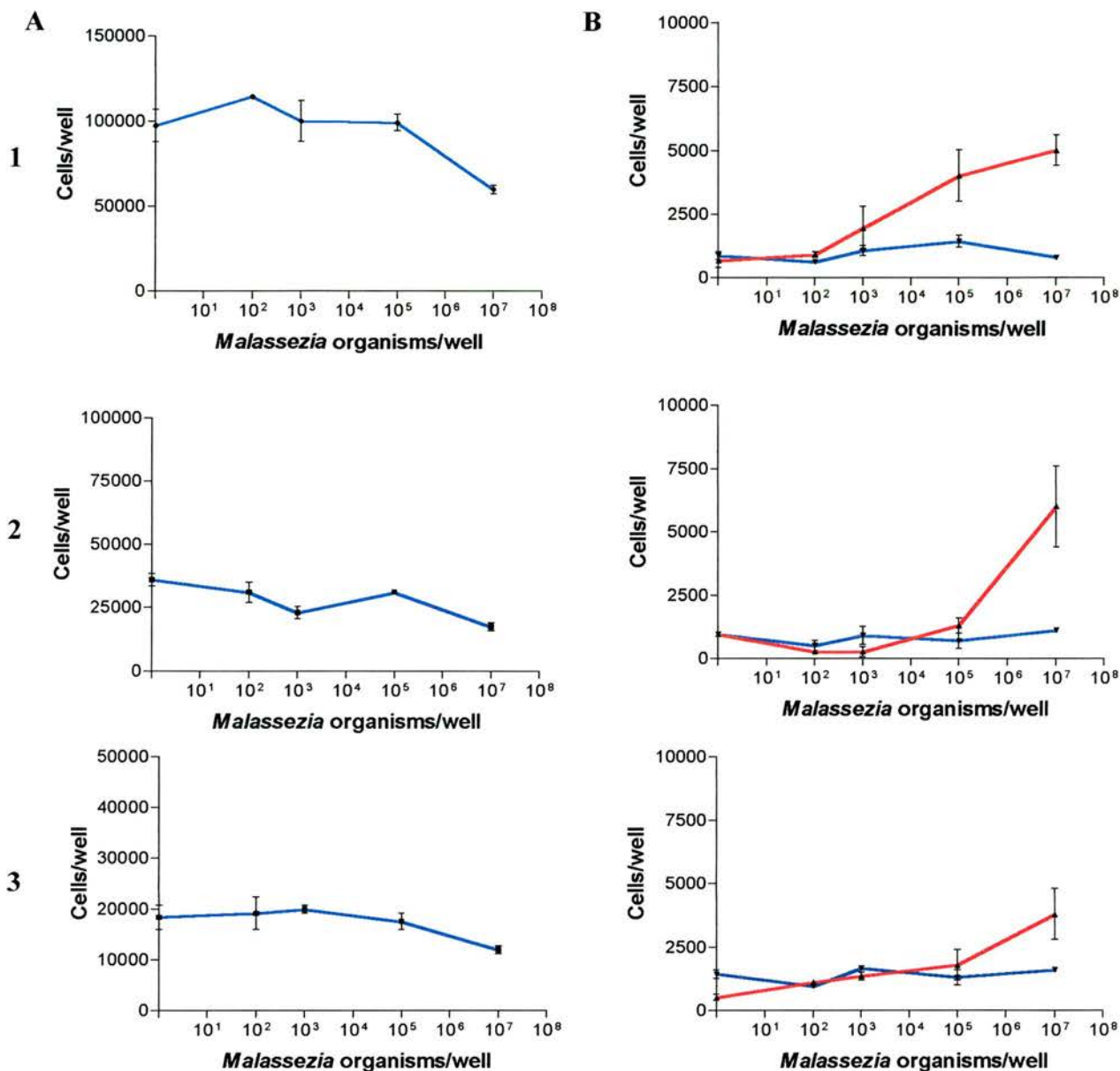


Figure 6.4.1 A-B Cell counts of keratinocytes co-cultured with *Malassezia pachydermatis* organisms for one day

A: The results obtained from three repeated experiments (1-3) showing the number of viable keratinocytes in the monolayer after co-culture with or without 10^2 , 10^3 , 10^5 , and 10^7 *Malassezia* organisms for one day. A prominent decrease in cell number was detected when the keratinocyte monolayer was co-cultured with 10^7 *Malassezia* organisms. B: The results obtained from three repeated experiments showing the number of cells in the medium after co-culture with or without 10^2 , 10^3 , 10^5 , and 10^7 *Malassezia* organisms for one day. In contrast to the monolayers, higher numbers of dead cells tended to be present in the medium when keratinocytes were co-cultured with higher numbers of *Malassezia* organisms (Red lines), whereas the number of viable cells in the medium was not dramatically affected by co-culture (Blue lines). Each data point represents the mean and standard error of the mean of duplicates.

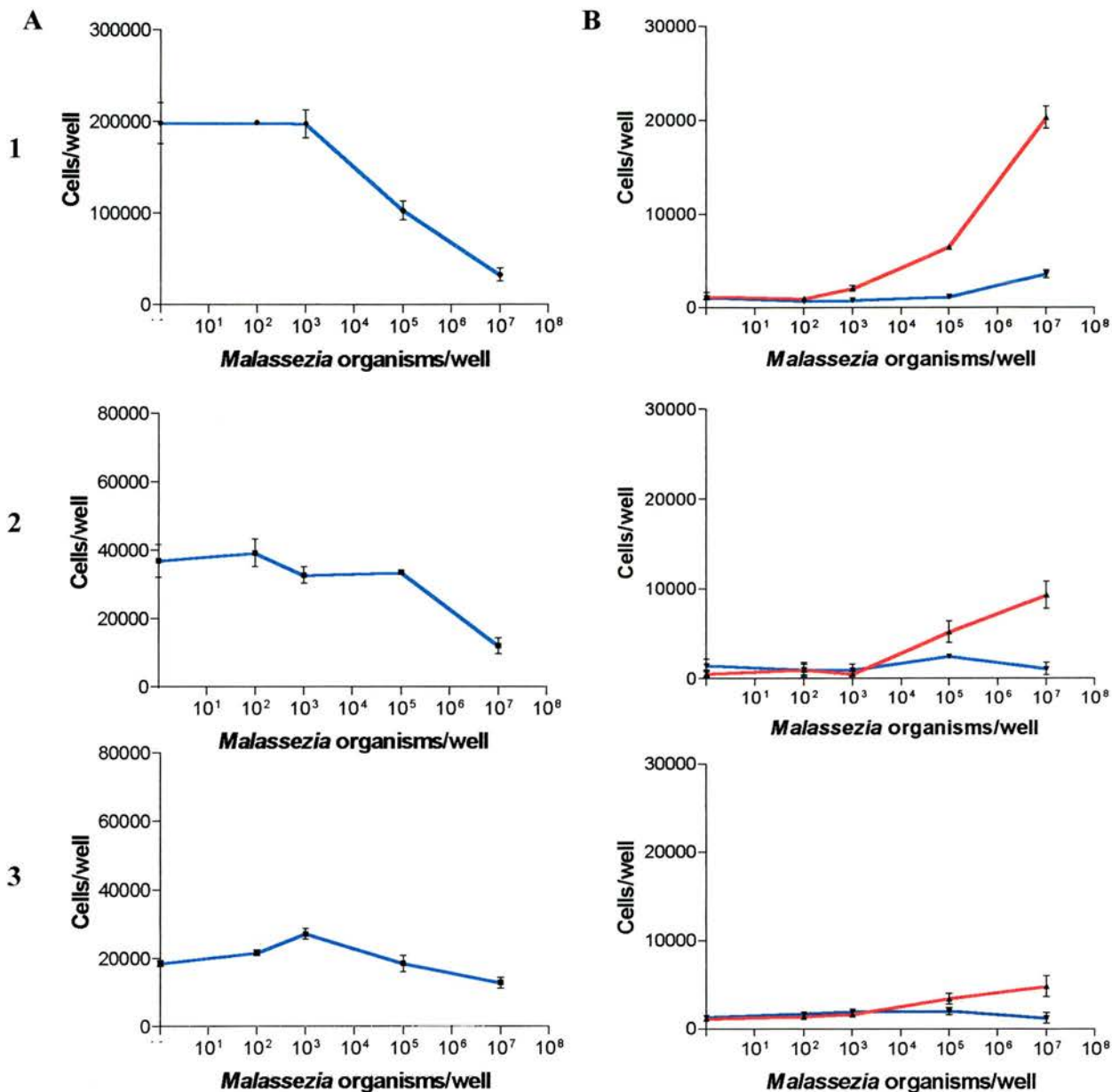


Figure 6.4.2 A-B Cell counts of keratinocytes co-cultured with *Malassezia pachydermatis* organisms for two days

A: The results obtained from three repeated experiments (1-3) showing the number of viable keratinocytes in the monolayer after co-culture with or without 10², 10³, 10⁵, and 10⁷ *Malassezia* organisms for two days. Keratinocytes cultured with higher numbers of *Malassezia* organisms tended to have lower numbers of cells in the monolayer. B: The results obtained from three repeated experiments showing the number of cells in the medium after co-culture with or without 10², 10³, 10⁵, and 10⁷ *Malassezia* organisms for two days. The number of dead cells in the medium rose as the number of *Malassezia* organisms increased (Red lines). Also, the number of viable cells in the medium remained relatively consistent (Blue lines), compared to that of dead cells. Each data point represents the mean and standard error of the mean of duplicates.

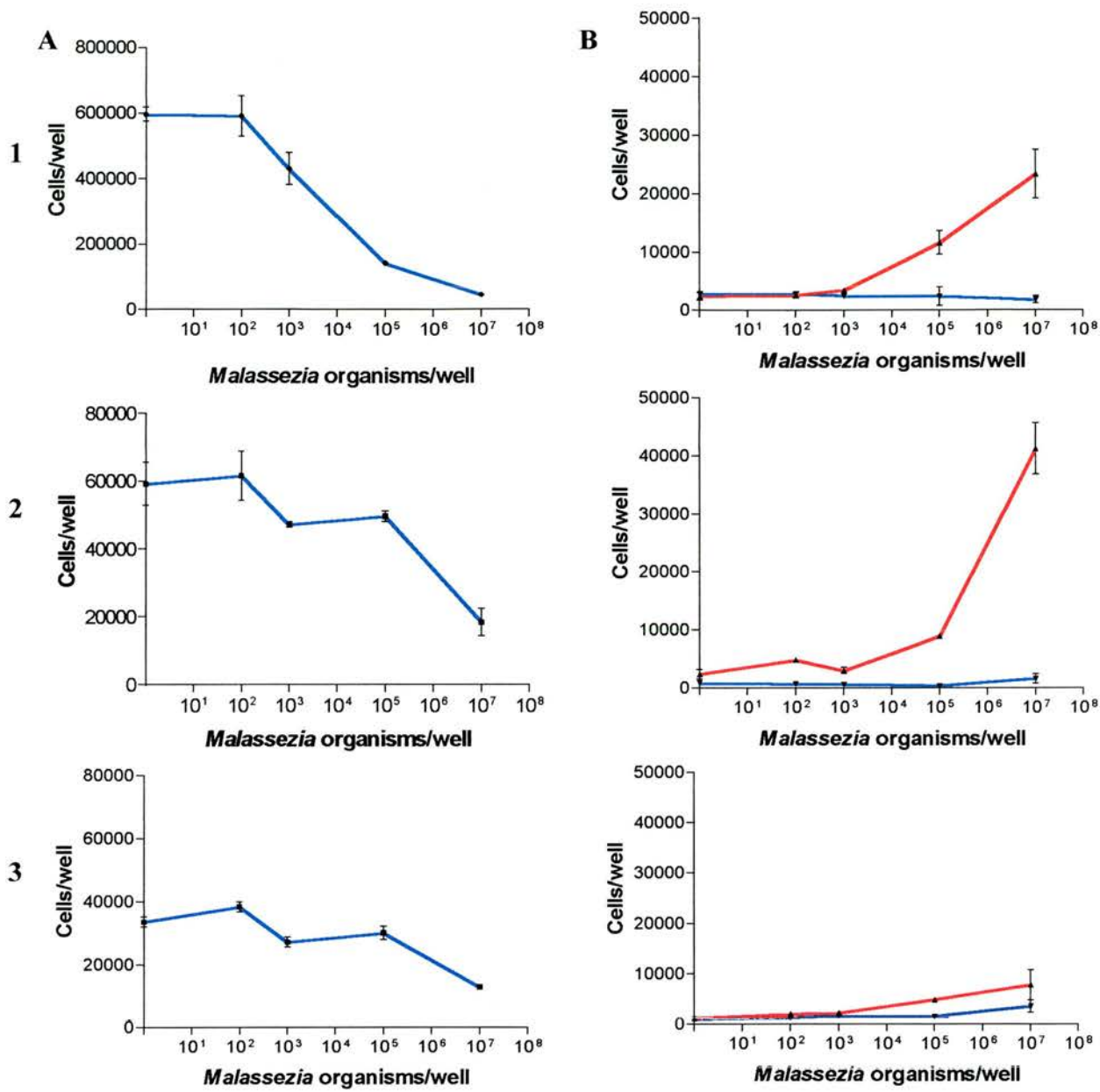


Figure 6.4.3 A-B Cell counts of keratinocytes co-cultured with *Malassezia pachydermatis* organisms for three days

A: The results obtained from three repeated experiments (1-3) showing the number of viable keratinocytes in the monolayer after co-culture with or without 10², 10³, 10⁵, and 10⁷ *Malassezia* organisms for three days. A decrease in cell numbers was detected in the groups co-cultured with higher numbers of *Malassezia* organisms compared to the control. B: The results obtained from three repeated experiments showing the number of cells in the medium after co-culture with or without 10², 10³, 10⁵, and 10⁷ *Malassezia* organisms for three days. As in Figures 6.4.1 B and 6.4.2 B, higher numbers of dead cells were present in the medium as the number of *Malassezia* organisms increased, whilst the number of viable cells was relatively unaffected, irrespective of the density of yeast cells. Each data point represents the mean and standard error of the mean of duplicates. Red line: dead cells; Blue line: viable cells.

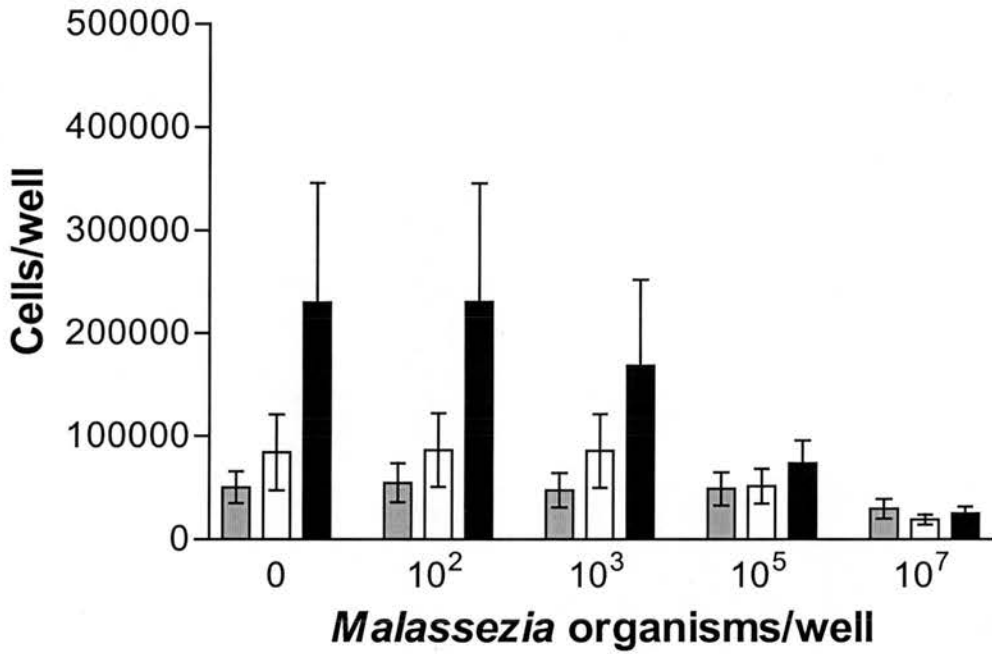


Figure 6.4.4 Comparison of the proliferation rate of keratinocytes co-cultured with *Malassezia pachydermatis*

Combined data from three repeated experiments shown in Figure 6.4.1 A, 6.4.2 A, and 6.4.3 A. Rapid growth was seen in keratinocytes cultured without *Malassezia* organisms and those co-cultured with low numbers of yeast cells on the last day of co-culture. In contrast, keratinocytes co-cultured with high numbers of *Malassezia* organisms only exhibited limited growth during three-day co-culture. Each bar represents the mean and standard error of the mean of six replicates. Shaded bars: day 1 of co-culture, Open bars: day 2, Black bars: day 3.

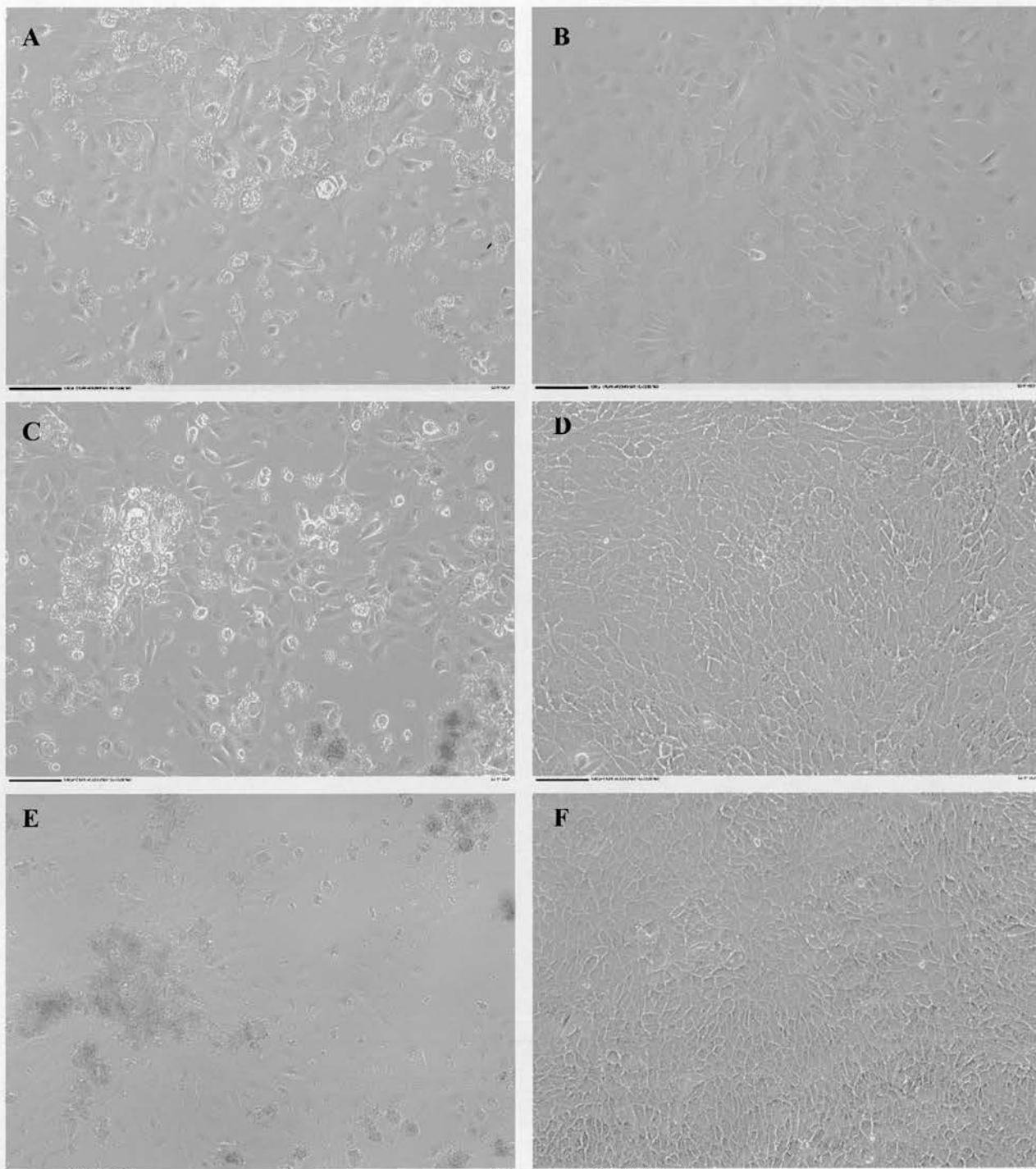


Figure 6.5 A-F Morphology of canine keratinocyte cultures co-cultured with or without *Malassezia pachydermatis* organisms (x100)

A, C, and E: Keratinocytes co-cultured with 10^7 *Malassezia* organisms for one, two and three days, respectively. No apparent increase in cell density was observed during three-day co-culture. The keratinocyte culture showed abnormal morphology in E.

B, D, and F: Keratinocytes cultured without *Malassezia* organisms served as controls for A, C, and E, respectively. Nearly confluent culture is shown in D, and the culture in F exhibited even denser cell populations with some cells being smaller than others.

Scale bars = 100 μ m.

IMMUNOHISTOCHEMISTRY STAINING OF CANINE KERATINOCYTES

CO-CULTURED WITH *MALASSEZIA PACHYDERMATIS*

6.4 Detection of proliferation markers in canine keratinocyte cultures co-cultured with *Malassezia pachydermatis*

Aims

No stimulatory effect of *Malassezia* organisms on the proliferation of canine keratinocytes was demonstrated in the previous section within three days of co-culture. The aim of the experiments in this section was to assess the proliferation rate of keratinocytes co-cultured with *Malassezia* organisms within 24 hours of co-culture, using immunohistochemistry techniques with antibodies against two proliferation markers, PCNA and Ki-67.

Methods

Keratinocytes were washed, resuspended in complete WME and counted in a haemocytometer. They were then plated into 8-well chamber slides at 2×10^4 viable cells/cm² (1.4×10^4 cells/well), and incubated at 37°C with 5 % CO₂ in air. After 24 hours, the medium was replaced with complete WME without PSF and the slides were incubated for a further three days to allow monolayer formation. On day 5, the medium was changed again and the monolayers were co-cultured with *Malassezia* organisms at similar densities to the experiments in the former sections. A volume of 100 µl of *Malassezia* suspension prepared as described in section 6.2.1 and containing ten fold dilutions from 2×10^6 to 2×10^2 yeast cells per well was added to wells containing growing keratinocytes and 300 µl of medium. Keratinocytes cultured in 400 µl of medium without *Malassezia* organisms served as the controls. At 4, 12, 16 and 24 hours post-co-culture, the cells were fixed with 4 %

paraformaldehyde as described in section 2.21 and the slides were stored at 4°C in 70 % alcohol until used. The immunohistochemistry staining of PCNA and Ki-67 was carried out following the procedures described in section 2.28.1 and 2.28.2, respectively. The proliferation rate was determined by counting the number of positively stained cells out of 100 in each chamber (section 2.29) and the positive cells were subjectively graded into two groups depending on the strength of staining.

6.4.1 Proliferating cell nuclear antigen detection

Results

Figure 6.6 A shows an example of PCNA labeled canine keratinocytes after the monolayer was co-cultured with 2×10^3 *Malassezia* organisms for 16 hours. Positively stained cells showed brown staining nuclei whereas negative cells appeared blue, the colour of the counterstain. All cells stained blue in the negative control slide (Figure 6.6 B). Between 80–90 % of the keratinocytes counted stained positively for PCNA, regardless of the density of *Malassezia* organisms used and at all time points (Figure 6.7 A-D). Furthermore, the number of strongly and weakly labeled positive cells did not differ between groups. The results indicated that *Malassezia* organisms did not affect PCNA expression in canine keratinocytes within 24 hours of co-culture.

6.4.2 Ki-67 antigen detection

Results

The Ki-67 staining of canine keratinocytes is shown in Figure 6.8. Positively labeled cells stained brown in comparison to unstained cells exhibiting blue staining (Figure 6.8 A) and cells in the negative control slide (Figure 6.8 B). Keratinocytes stained

with anti-Ki-67 Ab revealed similar results to those of PCNA staining. Of the 100 keratinocytes counted, approximately 80–90 % of the cells were positively stained in the monolayers co-cultured with or without various densities of *Malassezia* organisms at 4, 12, 16 and 24 hours post-co-culture (Figure 6.9 A-D). However, keratinocytes cultured with 2×10^6 *Malassezia* organisms showed a slightly lower proliferation rate after 12 hours. Also, fewer strongly labeled cells tended to be present in these keratinocytes compared to the other groups, which might explain the low numbers of keratinocytes detected in the monolayers co-cultured with the highest density of *Malassezia* organisms in section 6.3. These results indicated that *Malassezia* organisms did not affect Ki-67 expression in canine keratinocytes within 24 hours of co-culture, and further confirmed that *Malassezia* organisms did not stimulate proliferation of canine keratinocytes.

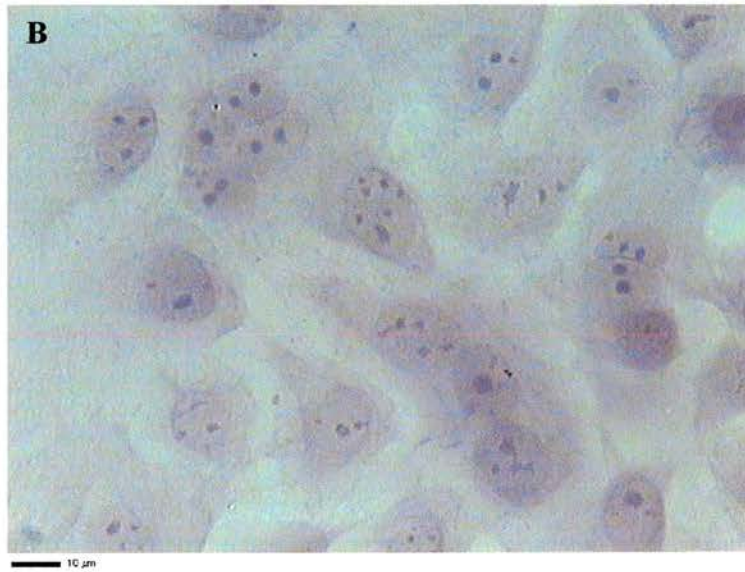


Figure 6.6 A-B PCNA staining in canine keratinocytes co-cultured with *Malassezia pachydermatis* organisms (x400)

A: PCNA staining in a keratinocyte monolayer co-cultured with 2×10^3 *Malassezia* organisms for 16 hours. Keratinocytes reacting positively with anti-PCNA Ab showed brown staining. Negatively stained cells appeared blue, the colour of the counterstain. B: Keratinocytes stained with Ab negative control. All cells were negatively stained.

Scale bars = 10 μm.

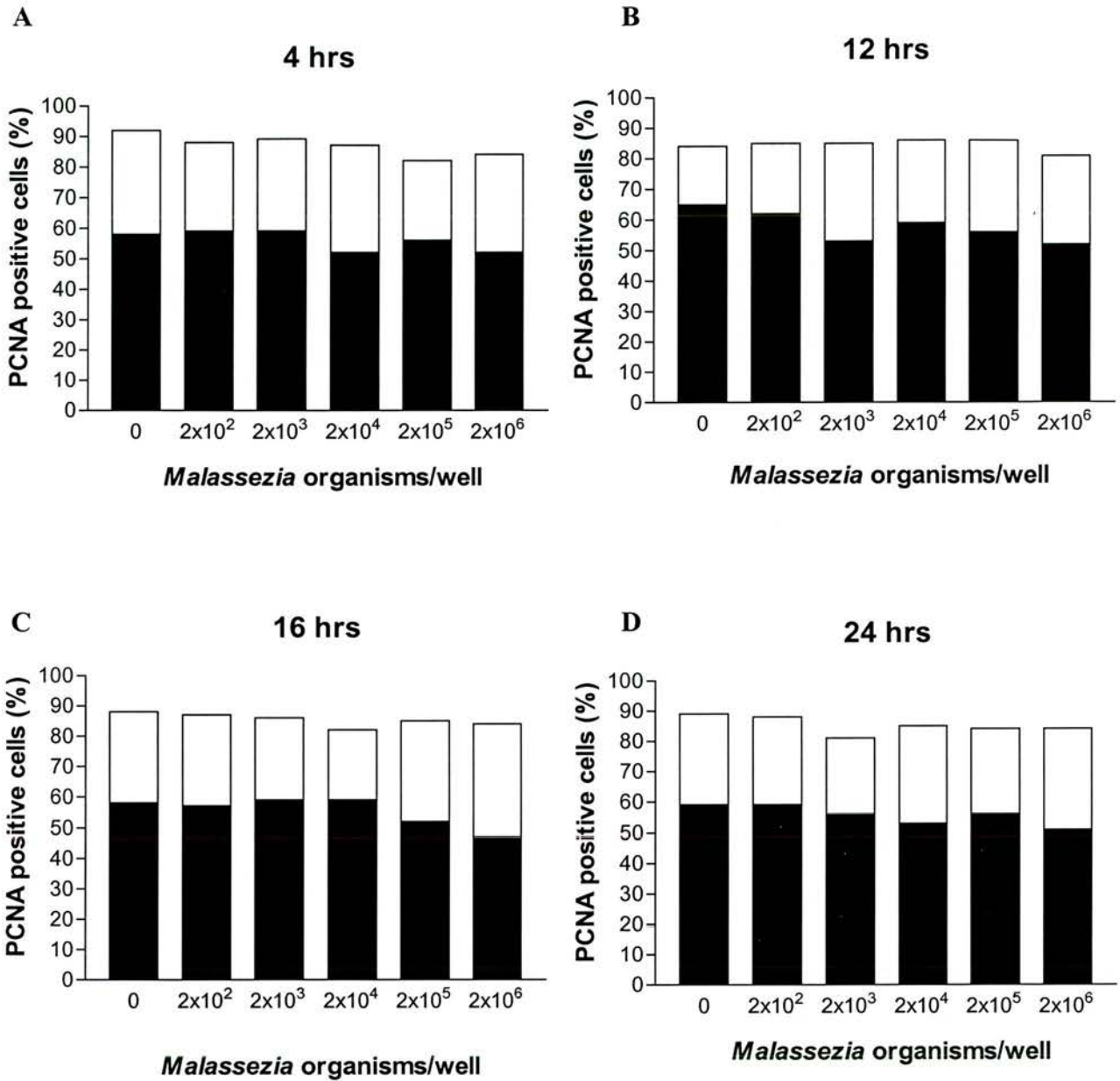


Figure 6.7 A-D PCNA-positive canine keratinocytes co-cultured with *Malassezia pachydermatis* organisms

A: PCNA-positive keratinocytes detected at 4 hours post-co-culture. B: 12 hours; C: 16 hours; D: 24 hours. Approximately 80-90 % of the cells counted were positively stained with PCNA in keratinocyte monolayers co-cultured with or without *Malassezia* organisms, irrespective of the density of yeast cells used. The number of strongly and weakly labeled cells was similar, indicating that *Malassezia pachydermatis* had no effect on PCNA expression in canine keratinocytes. Black bars: strongly labeled cells; Open bars: weakly labeled cells.

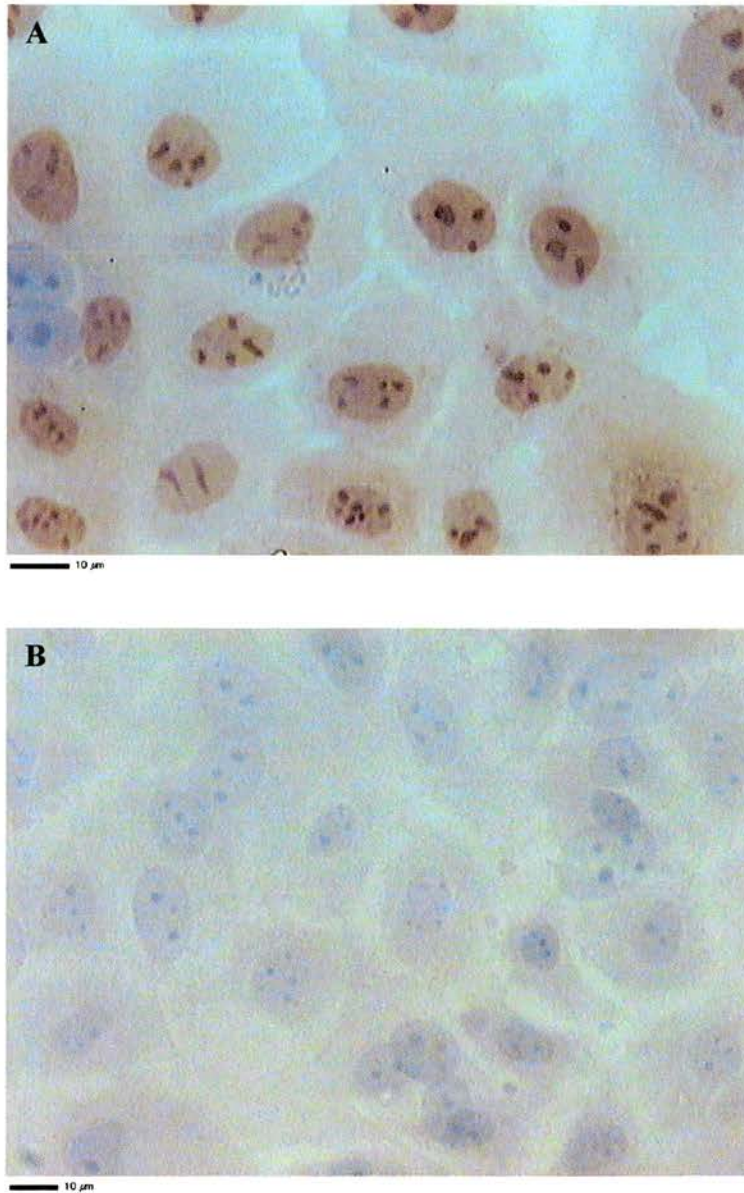


Figure 6.8 A-B Ki-67 staining in canine keratinocytes co-cultured with *Malassezia pachydermatis* organisms (x400)

A: Ki-67 staining in a keratinocyte monolayer co-cultured with 2×10^3 *Malassezia* organisms for 16 hours. Keratinocytes reacting positively with anti-Ki-67 Ab showed brown staining. B: Keratinocytes stained with Ab negative control. All cells appeared unstained.

Scale bars = 10 μm.

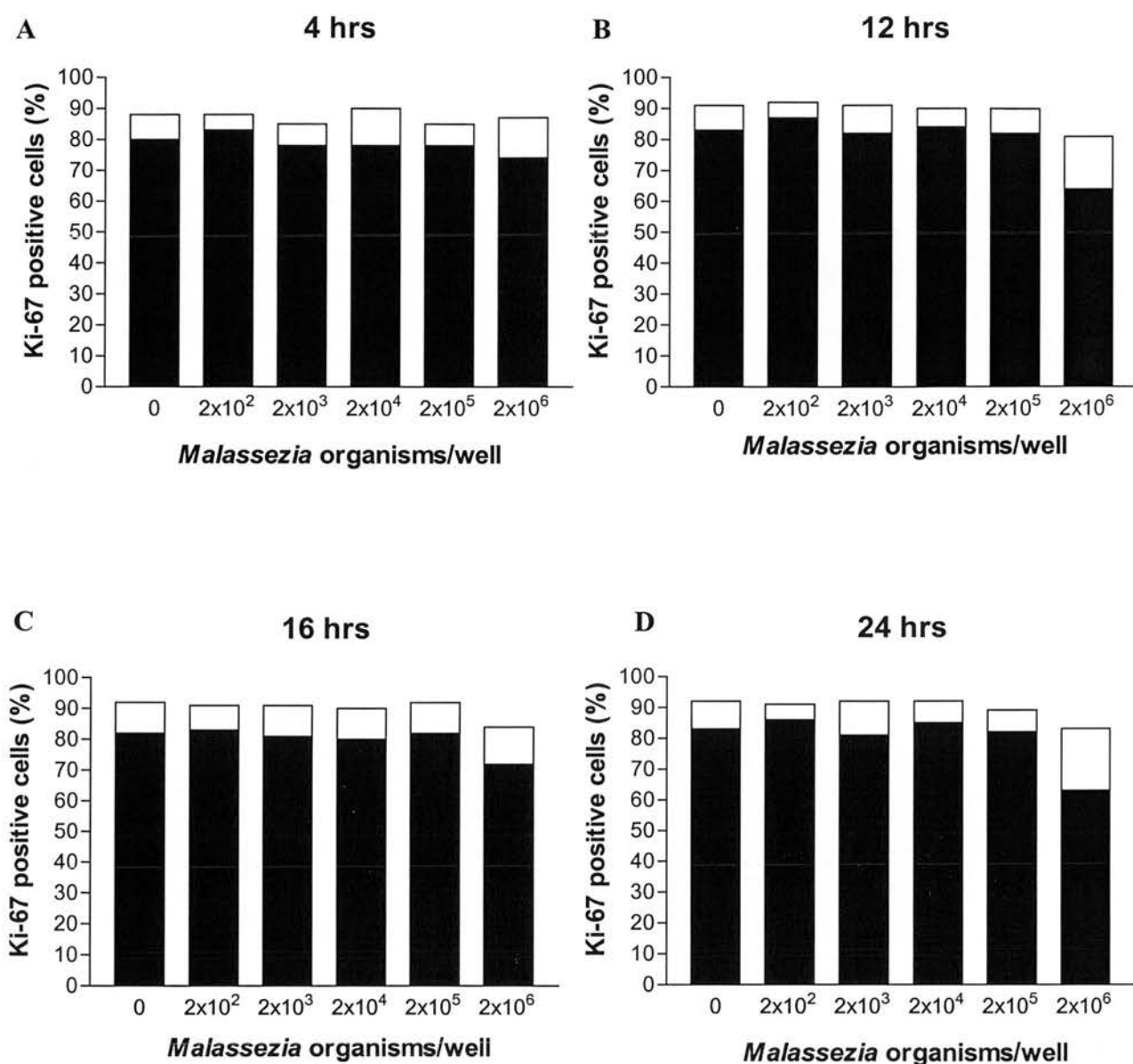


Figure 6.9 A-D Ki-67 positive canine keratinocytes co-cultured with *Malassezia pachydermatis* organisms

A: Ki-67 positive keratinocytes detected at 4 hours post-co-culture. B: 12 hours; C: 16 hours; D: 24 hours. Approximately 80-90 % of the cells counted were positively stained with Ki-67 in keratinocyte monolayers co-cultured with or without *Malassezia* organisms, irrespective of the density of yeast cells used. The results indicated that *Malassezia pachydermatis* did not affect Ki-67 expression in canine keratinocytes within 24 hours of co-culture. The keratinocyte monolayers co-cultured with 2×10^6 *Malassezia* organisms tended to have fewer strongly labeled cells. Black bars: strongly labeled cells; Open bars: weakly labeled cells.

6.5 Detection of cell death in canine keratinocytes co-cultured with *Malassezia pachydermatis*

Aims

In section 6.3, keratinocyte monolayers co-cultured with high numbers of *Malassezia* organisms were shown to have high numbers of dead cells in the medium. In order to investigate the possibility that *Malassezia* organisms caused cell death, an apoptosis detection kit was used to label keratinocytes co-cultured with *Malassezia* organisms in this experiment.

Methods

Cultivation of keratinocytes on chamber slides and *Malassezia* co-culture were carried out as described in section 6.4. Six chamber slides containing keratinocyte cultures were prepared and the keratinocyte monolayers were co-cultured with five different densities of *Malassezia* organisms (2×10^6 to 2×10^2 yeast cells per well). Keratinocytes cultured without *Malassezia* organisms acted as controls. At 4, 12, 16, 24, 48 and 72 hours post-co-culture, the slides were fixed with 4 % paraformaldehyde, and stored at 4°C in 70 % alcohol until used. The apoptosis detection was performed using DermaTacs™, an *in situ* apoptosis detection kit for skin cells and tissues (R & D Systems Europe, Oxon, UK), and the manufacturer's instructions were followed (see section 2.28.3 for details). The whole area of a well on the chamber slide was scanned for apoptotic cells following the criteria described in section 2.29.

Results

Figure 6.10 A-C shows a representative apoptotic cell identified using the apoptosis

detection kit as well as the positive and negative controls. The positively stained cell in Figure 6.10 A shows blue staining in the nucleus, indicating that it contained fragmented nuclear chromatin characteristic of apoptosis, detected by the labelling process mediated by TdT enzyme. The positive control slide was treated with nuclease and all cells showed blue nuclear staining (Figure 6.10 B). No blue stained cells were present in the negative control slide which was generated by omitting TdT enzyme in the staining procedure (Figure 6.10 C).

The results obtained at 4, 12, 16, 24, 48 and 72 hours post-co-culture are shown in Figure 6.11 A-F. More apoptotic cells were present in keratinocyte monolayers co-cultured with 2×10^5 or 2×10^6 *Malassezia* organisms as compared with the control or cells co-cultured with lower densities of *Malassezia* within 24 hours of co-culture. The number of apoptotic cells in monolayers co-cultured with 2×10^2 to 2×10^4 *Malassezia* organisms also gradually increased during this period of time. At 48 hours post-co-culture, the monolayer co-cultured with 2×10^3 *Malassezia* organisms had the highest number of apoptotic cells, and the number of these cells continued to increase between 48 and 72 hours post-co-culture. In contrast, lower numbers of apoptotic cells were detected in keratinocytes co-cultured with 2×10^5 or 2×10^6 *Malassezia* organisms at 48 and 72 hours post-co-culture. However, far fewer cells were observed to be attached to the slides in these two groups, which might explain the low numbers of apoptotic cells detected. The results of this experiment suggested that *Malassezia* organisms appeared to induce canine keratinocytes to undergo cell death.

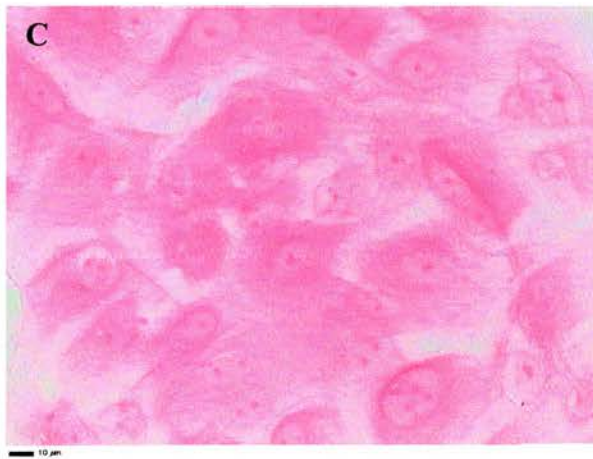
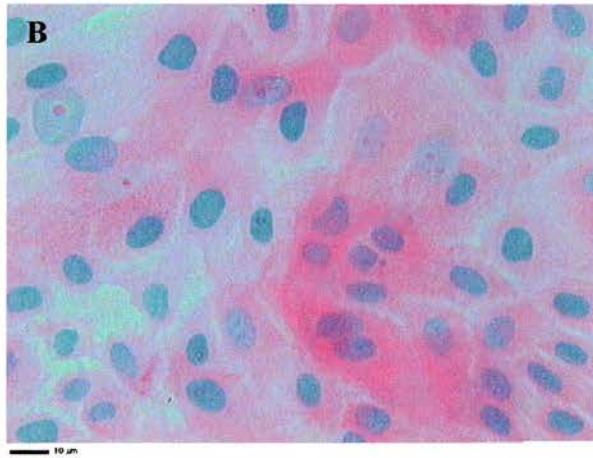
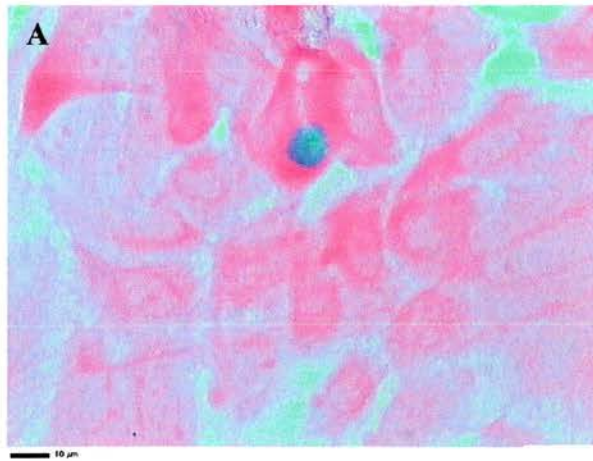


Figure 6.10 A-C Apoptosis detection in canine keratinocytes co-cultured with *Malassezia pachydermatis* organisms

A: An apoptotic cell (x400). The cell showed blue nuclear staining and pink cytoplasm. B: Positive control (x250). All cells exhibited blue stain in their nuclei due to the use of nuclease. C: Negative control (x250). No blue staining was present and all cells stained pink, the colour of the counterstain.

Scale bars = 10 μm.

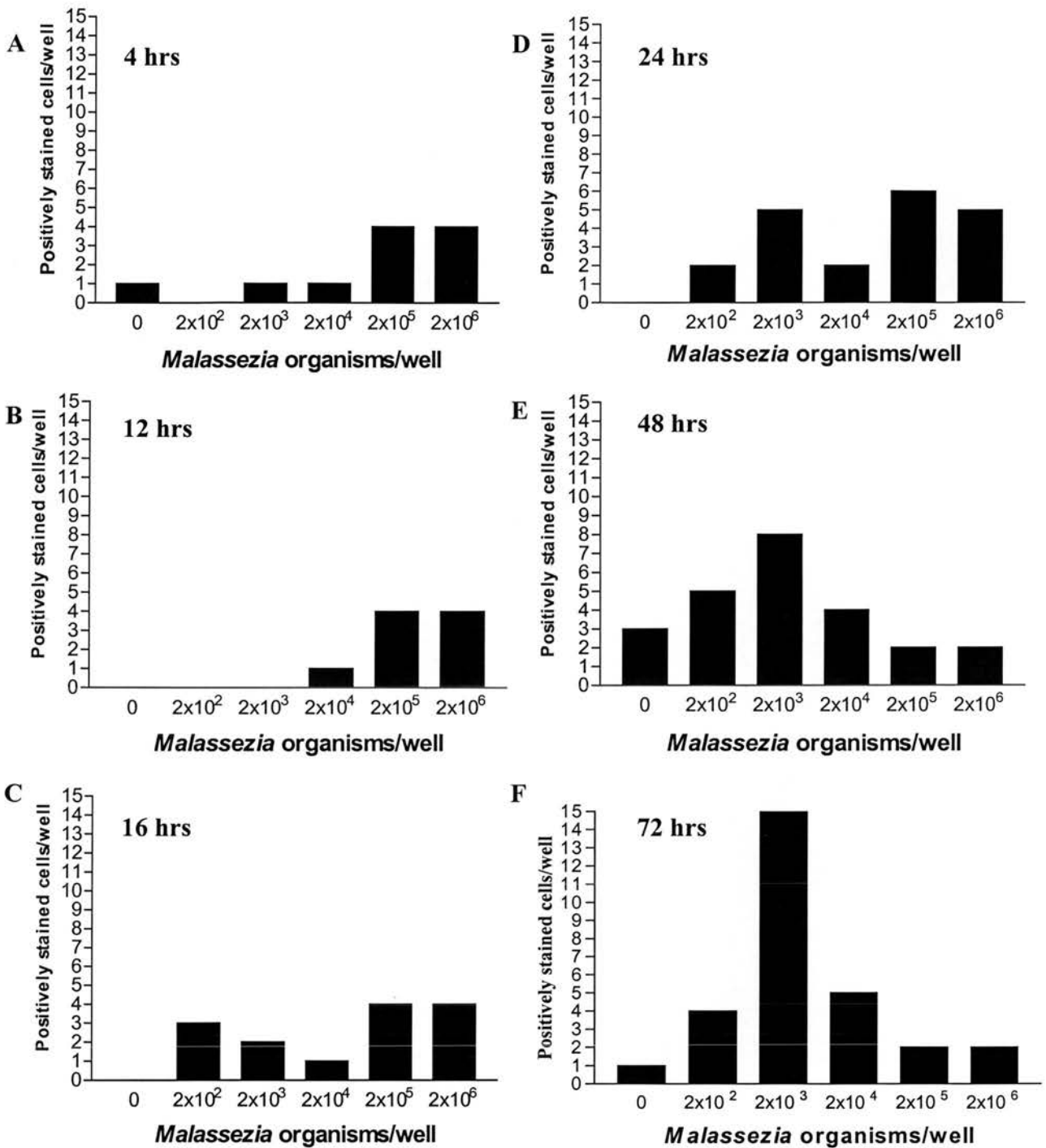


Figure 6.11 A-F Induction of cell death in canine keratinocytes by *Malassezia pachydermatis*

A: The number of apoptotic cells detected at 4 hours post-co-culture. B: 12 hours; C: 16 hours; D: 24 hours; E: 48 hours; F: 72 hours. More apoptotic cells were present in the keratinocyte monolayers co-cultured with 2×10^5 or 2×10^6 *Malassezia* organisms within 24 hours of co-culture. At 48 and 72 hours post-co-culture, the highest number was detected in the groups co-cultured with 2×10^3 *Malassezia* organisms, whereas the monolayers co-cultured with 2×10^5 or 2×10^6 yeast cells exhibited lower numbers of apoptotic cells, which might be due to the considerably fewer keratinocytes attached to the slides compared to the other groups. The increasing numbers of apoptotic cells detected in keratinocyte monolayers during three-day co-culture with *Malassezia* organisms indicated that *M. pachydermatis* appeared to induce death of canine keratinocytes.

6.6 Discussion

6.6.1 Assessment of a cell proliferation assay for use with canine keratinocytes co-cultured with *Malassezia pachydermatis*

The initial aim of this part of the study was to assess the feasibility of using a colourimetric cell proliferation assay, described in the previous chapter, to evaluate the proliferation of cultured canine keratinocytes co-cultured with *Malassezia pachydermatis* organisms. The results shown in section 6.2.1 demonstrated that *Malassezia* organisms reacted with the assay reagent and generated detectable signals, especially with high numbers of yeast cells. Although the signals generated by *Malassezia* organisms alone were treated as background and subtracted from the wells containing growing keratinocytes and *Malassezia* organisms, the results were not in accordance with microscopic observation. It appeared that the assay overestimated the number of keratinocytes that were actually present in the wells and this was particularly apparent in the groups co-cultured with high numbers of *Malassezia* organisms. The specific reason for this discrepancy was not known. One explanation could be that *Malassezia* organisms provoke changes in cell components that are not reflected by changes in cell number. As was mentioned in section 5.7.1, this colourimetric assay is based on the bioreduction of a tetrazolium salt to a coloured formazan. The process is mediated by a variety of intracellular dehydrogenases and mitochondrial dehydrogenases such as succinic dehydrogenase that is tightly bound to the mitochondrial inner membrane (reviewed in Marshall *et al.* 1995). Increased formazan production by cells has been observed in studies investigating the effects of interferons and cytotoxicity of anti-tumour drugs, even when the overall cell numbers decrease. The investigators concluded that this was attributed to enhanced mitochondrial activity in surviving cells (Jabbar *et al.* 1989;

Pagliacci *et al.* 1993). A similar reaction might happen in canine keratinocytes co-cultured with high numbers of *Malassezia pachydermatis* organisms, possibly indicating increased activity of mitochondria in keratinocytes, but not an increase in cell numbers. A second explanation is that changes in the pH of the medium might be responsible for the discrepancy. The enzyme kinetics, redox potentials of tetrazolium salts and absorption spectra of formazans within the assay process can all be sensitive to pH changes and as a result, pH shifts could distort dose-response curves observed in tetrazolium assays (Marshall *et al.* 1995). It is possible that the pH value of the medium containing growing keratinocytes and *Malassezia* organisms was different from that containing *Malassezia* organisms alone. Thus, the activities of keratinocytes used to generate the standard curve might not represent those of the cells co-cultured with *Malassezia* organisms, and the activities of *Malassezia* organisms alone could not reflect those co-cultured with keratinocytes. Due to these pitfalls of the assay system, this method was deemed to be unsuitable for use in the *in vitro Malassezia pachydermatis*-canine keratinocyte co-culture system and was therefore abandoned.

In order to eliminate the above problems, a direct cell counting technique was developed. The advantages of this method include easy discrimination between keratinocytes and yeast cells, individual quantification of keratinocytes in the monolayer or the medium, and minimal interference with biochemical or environmental factors due to its direct measurement of cell numbers. However, the manual counting procedure is laborious and more likely to suffer from investigator bias. Also, it is almost inevitable that some cells are lost during the trypsinisation step required for attached cell cultures. This renders the direct cell counting

technique prone to more technical errors than biochemical assays, such as the colourimetric cell proliferation assay tested in section 6.2 which does not require a trypsinisation step. Furthermore, the direct counting technique is less suitable for processing large numbers of samples. It is possible that other microculture assays might be suitable for the *in vitro* yeast-keratinocyte system. One example is a colourimetric assay that is less sensitive to environmental fluctuations and based upon a stoichiometric association between a protein binding dye, sulforhodamine B (SRB), and the protein content of the cell, in contrast to the enzymatically driven tetrazolium assays (Rubinstein *et al.* 1990; Skehan *et al.* 1990). In a SRB assay, cultures are fixed with trichloroacetic acid (TCA) before staining with SRB dissolved in 1 % acetic acid. The SRB then binds electrostatically to basic amino acid residues of proteins in fixed cells under mildly acidic conditions. After unbound dye is removed by washing with 1 % acetic acid, the protein-bound dye is extracted from cells and solubilised for optical density measurement by a weak base such as 10 mM Tris. The SRB assay offers several advantages over tetrazolium assays. By adding TCA at elevated concentrations into a single cell suspension in which cells have been allowed to settle down, it promotes cell attachment to the plastic substratum, which can be useful for adherent cultures that shed floating cells or small aggregates into the medium. The assay procedure is less sensitive to environmental fluctuations such as pH change, and is independent of intermediary metabolism. Additionally, it generates a stable colourimetric end point that does not have to be measured within any fixed period of time (Skehan *et al.* 1990). However, careful validation would be required before it could be applied to co-cultures of canine keratinocytes and *Malassezia* organisms.

6.6.2 The effect of *Malassezia pachydermatis* on cultured canine keratinocytes

The experiments performed using viable *Malassezia pachydermatis* organisms in this chapter followed on from those in the previous chapter using extracts and culture supernatants. Similar to the findings presented in the previous chapter, the results in section 6.3 showed that *Malassezia* organisms did not cause canine keratinocyte proliferation. These results have two implications. They could indicate that *M. pachydermatis* has no direct effect on keratinocyte proliferation either *in vitro* or *in vivo*. If this is the case, the epidermal hyperplasia is likely to be due to the inflammatory reaction stimulated by the organism itself or the underlying diseases. Alternatively, *M. pachydermatis* has no direct effect in this *in vitro* system, but could have a direct effect in a living epidermis with a blood supply and a functional immune system. This possibility can be supported by the studies on *Candida albicans* which have demonstrated increased epidermal proliferative responses to this fungus *in vivo* using experimental animals (Sohnle & Kirkpatrick 1978; Sohnle & Hahn 1989). Furthermore, the epidermal proliferation appeared to be enhanced by the host's immunological reactions to experimental cutaneous candidiasis (Sohnle & Kirkpatrick 1978; Wilson & Sohnle 1986).

The dose-dependent increase in dead keratinocytes in the culture medium indicated that *Malassezia pachydermatis* had a cytotoxic effect on canine keratinocytes *in vitro*. Various mechanisms might explain why keratinocytes detached from the culture plates and shed into the medium. *Malassezia* organisms might affect the function of transmembrane adhesion proteins of keratinocytes. The counting of cells in the monolayers treated with high numbers of *Malassezia* organisms (10^5 or 10^7) revealed that the increase in cell numbers was minimal during a three day co-culture. Since

adhesion to the substratum is required for keratinocyte growth and loss of attachment would cause a rapid growth arrest (Gniadecki 1998), the increasing number of dead cells in the medium could be explained by the scenario that the cells in the monolayers treated with 10^5 or 10^7 *Malassezia* organisms were proliferating, but the adherence of *Malassezia* organisms and their interactions with keratinocytes damaged the ability of keratinocytes to adhere to the culture plate. However, the identity of such an adhesion molecule is not known and awaits further study. Secondly, the keratinocytes in the growing monolayers could simply undergo necrosis and then shed into the medium. A necrotic effect of *Malassezia furfur* at high ratios of yeast cells to human keratinocytes has been reported in a study investigating the effect of *Malassezia furfur* on a human keratinocyte cell line (Baroni *et al.* 2001a). Finally, *Malassezia pachydermatis* could be internalised by canine keratinocytes and induce keratinocyte apoptosis leading to cell detachment. Engulfment of *Malassezia furfur* has been shown with human keratinocytes (Baroni *et al.* 2001a). Invasion of human keratinocytes by *Staphylococcus aureus* has also been demonstrated to cause necrosis and apoptosis (Nuzzo *et al.* 2000; Mempel *et al.* 2002). Although keratinocyte apoptosis was not investigated in the study by Baroni *et al.* (2001a), it is possible that invasion of canine keratinocytes by *Malassezia pachydermatis* could cause keratinocytes to undergo apoptosis. In order to confirm this possibility, further studies using May-Grumwald Giemsa stain or electron microscopy (Baroni *et al.* 2001a; Baroni *et al.* 2001b) to demonstrate the internalisation of *M. pachydermatis* by canine keratinocytes are required.

The immunohistochemistry staining studies described in sections 6.4 and 6.5 were designed as preliminary studies due to time constraints. The results of the staining for

cellular proliferation markers, PCNA and Ki-67, showed that there was no prominent difference in expression of PCNA and Ki-67 between canine keratinocytes co-cultured with *Malassezia pachydermatis* and those cultured without, regardless of the number of *Malassezia* organisms used. This was not in agreement with the only previous report which demonstrated a significant increase in expression of Ki-67 in canine keratinocytes co-cultured with *M. pachydermatis in vitro* compared to keratinocytes cultured alone *in vitro* (von Tscharnner *et al.* 1999), despite using the same antibody. As the details of this study have not been reported in a peer-reviewed publication, it is currently difficult to provide possible explanations for the discrepancy.

In section 6.5, more apoptotic cells were observed in the keratinocyte monolayers co-cultured with high numbers of *Malassezia* organisms compared to those cultured without, and the number increased after prolonged incubation. Despite the decreased number of positively stained keratinocytes observed in the groups co-cultured with 2×10^5 or 2×10^6 *Malassezia* organisms at 48 and 72 hours post-co-culture, which might be explained by the decline in the number of attached cells, the results suggested that *Malassezia pachydermatis* could induce canine keratinocytes to undergo cell death *in vitro*. The biological significance of this effect remains open to question, since apoptosis is not a characteristic feature in histopathological sections from the skin of dogs with *Malassezia* dermatitis. Nevertheless, these results provided further evidence in support of the findings in section 6.3 that *Malassezia pachydermatis* could cause death of keratinocytes. Another explanation for the increase in the number of apoptotic cells in keratinocyte monolayers co-cultured with *Malassezia* organisms during a 3 day co-culture could be that the nutrients in the

medium were gradually depleted leading to cell deletion in the proliferating populations. In order to assess this speculation, other non-pathogenic microorganisms might be introduced into the *in vitro* system as controls.

In summary, the experiments in this chapter have demonstrated that *Malassezia pachydermatis* does not accelerate the proliferation of canine keratinocytes *in vitro*. However, the organisms appear to induce keratinocytes to detach from the monolayer and to undergo cell death. Future studies would involve further investigation of the role of *M. pachydermatis* in epidermal hyperplasia associated with *Malassezia* dermatitis using living epidermis (skin explants) and an air-medium interface culture system. Also, application of *Malassezia* organisms to the skin of experimental dogs, both normal and atopic, and investigation of the effect of *M. pachydermatis* on cytokine production and expression of adhesion molecules by canine keratinocytes would provide further insight into the role of *M. pachydermatis* as a pathogenic factor in the histopathology of *Malassezia* dermatitis in dogs.

Chapter 7

GENERAL DISCUSSION

Malassezia dermatitis, an inflammatory dermatosis associated with elevated cutaneous populations of *Malassezia pachydermatis*, has been recognised with increasing frequency in recent years, and dogs with atopic dermatitis are especially predisposed to the condition (reviewed in section 1.3.3). Atopic dogs have been found to have elevated levels of *Malassezia*-specific IgG and IgE in their sera (Nuttall & Halliwell 2001). Significantly greater intradermal test (IDT) reactivity to *M. pachydermatis* extracts has been observed in atopic dogs with cytological evidence of *Malassezia* overgrowth than in atopic dogs without excessive numbers of the organism (Morris *et al.* 1998). Significantly higher frequencies of positive IDT reactivity to *M. pachydermatis* extract has also been found in atopic dogs compared to healthy dogs (Bond *et al.* 2002a). Furthermore, anti-*Malassezia* IgE antibodies have been demonstrated to be functional in Type I hypersensitivity reactions using passive cutaneous anaphylaxis tests (Morris & DeBoer 2002). The data from these studies provide evidence that some atopic dogs can become sensitised to *M. pachydermatis*-derived allergens, and the possible immunological pathways for this sensitisation are reviewed in section 1.4.1.2. However, there remained a lack of information on IgG and IgE binding proteins from *M. pachydermatis* in atopic dogs.

In this study, the protein antigens that might trigger *M. pachydermatis*-specific IgG and IgE responses in atopic dogs with *Malassezia* dermatitis were detected using SDS-PAGE and Western blotting techniques. The experiments described in chapter 3 demonstrated a greater IgG response to *M. pachydermatis* in atopic dogs with

Malassezia dermatitis and identified a 25 kDa antigen that was recognised in the majority of these dogs. This 25 kDa antigen was only recognised by less than 25 % of the atopic dogs without *Malassezia* dermatitis and none of normal dogs, suggesting a degree of specificity for the IgG response to this antigen in atopic dogs with *Malassezia* dermatitis. The IgE immunoblots with sera from atopic dogs with *Malassezia* dermatitis and normal dogs revealed that the majority of the dogs in the former group had a greater IgE response to *M. pachydermatis* than normal dogs (chapter 4). Proteins with molecular weights of 45, 52, 56, and 63 kDa were recognised by over 50 % of the atopic dogs with *Malassezia* dermatitis, but only recognised by a minority of normal dog sera. Therefore, they can be classified as major allergens. These results suggest that *Malassezia* dermatitis in atopic dogs is associated with an IgG and IgE response to *M. pachydermatis*, which provides further evidence to support the hypothetical mechanisms stated in section 1.4.1.2. However, the identities of the proteins described above are not known and await further investigation.

In human medicine, some of the recombinant allergens (rMal s 1 and rMal s 5-9) have been tested for use in skin prick testing, atopy patch testing, and *in vitro* IgE assays (Zargari *et al.* 2001; Johansson *et al.* 2002). In order to improve the specificity of IDT with *M. pachydermatis* extracts in atopic dogs, which is currently performed only for research purposes (Morris *et al.* 1998; Bond *et al.* 2002a), future studies are planned to determine the identities of the 45, 52, 56, and 63 kDa allergens. The crude *Malassezia* extracts used in this study could be purified using high-performance liquid chromatography (HPLC) and separated by electrophoresis. The 45, 52, 56, and 63 kDa allergens recognised by the sera of atopic dogs with *Malassezia* dermatitis

could then be identified by N-terminal sequencing and further expressed as recombinant proteins by cloning techniques (Schmidt *et al.* 1997; Yasueda *et al.* 1998; Onishi *et al.* 1999; Lindborg *et al.* 1999; Rasool *et al.* 2000). The recombinant proteins could be tested for use in IDT and might improve the diagnosis of *M. pachydermatis*-associated Type I hypersensitivity in dogs with atopic dermatitis and *Malassezia* overgrowth. Furthermore, the treatment of *Malassezia* dermatitis is currently based on anti-fungal therapy (reviewed in section 1.3.3.3). The use of recombinant proteins could provide an alternative therapeutic approach to *Malassezia* dermatitis in atopic dogs: allergen-specific immunotherapy. Further studies on the efficacy of immunotherapy with *Malassezia* allergens would yield data with therapeutic implications.

The role of the IgG response to the 25 kDa antigen in atopic dogs with *Malassezia* dermatitis was not investigated in this study. The 25 kDa antigen could also be further characterised using protein purification and sequencing techniques. The purified antigen could be used to investigate cell-mediated immune responses to *M. pachydermatis* such as cytokine production as T-cell mediated immune responses have been suggested to be important in host immunity against superficial mycoses (Hay 1992). Additionally, it could be used to study the capability of *M. pachydermatis* to activate complement by the classical and alternative pathways, allowing comparison with *Pityrosporum orbiculare* and *P. ovale* (Belew *et al.* 1980; Sohnle & Collins-Lech 1983). Such studies in the future would improve our understanding of the interaction between *M. pachydermatis* and the immune system of its canine host.

In addition to activation of the skin immune system, the skin also protects itself from environmental insults by thickening the epidermis. Epidermal hyperplasia is one of the characteristic features seen in histopathology of *Malassezia* dermatitis (section 1.3.3.3.2). Little is known about the direct interaction between *Malassezia* organisms and canine keratinocytes but a preliminary report had demonstrated a significant increase in a cellular proliferation marker in canine keratinocytes co-cultured with *M. pachydermatis* compared to control cells *in vitro* (von Tscherner *et al.* 1999). In order to further investigate the potentially pathogenic role of *M. pachydermatis* in epidermal hyperplasia associated with *Malassezia* dermatitis, studies were designed to evaluate the proliferation of canine keratinocytes co-cultured with extracts and culture supernatants from *M. pachydermatis* (chapter 5) and live *Malassezia* organisms (chapter 6). The experiments in chapter 5 showed that *Malassezia* extracts, either with or without protease inhibitors, and culture supernatants from the yeast, did not affect the proliferation of normal canine keratinocytes *in vitro*. Likewise, *Malassezia* organisms did not cause canine keratinocyte proliferation *in vitro* (chapter 6). However, they induced keratinocytes to detach from the substratum and to undergo cell death. Although the biological significance of these effects is not yet clear, these results indicate that *M. pachydermatis* has no direct effect on proliferation of canine keratinocytes *in vitro*.

Nevertheless, it is possible that *M. pachydermatis* organisms might have a direct effect on the living epidermis with a blood supply and a functional immune system. Also, the comparison of the proliferative responses of keratinocytes from atopic dogs or dogs with *Malassezia* dermatitis to that of normal dogs was not addressed in this study. Skin from these dogs might react differently to *M. pachydermatis*. These

questions could be addressed by repeating the keratinocyte proliferation studies using cells obtained from affected dogs but it is unlikely that sufficient keratinocytes could be acquired for such experiments. The use of skin explants or *in vitro* epidermal culture systems based on an air-medium interface might allow further refinement of the experimental methodology. *In vivo* experiments involving application of live *Malassezia* organisms and extracts to the skin of dogs would also provide further information on the pathological processes involved.

Alternatively, *M. pachydermatis* might have no direct effect on keratinocyte proliferation, either *in vitro* or *in vivo*. If this is the case, the epidermal hyperplasia is likely to be due to the inflammatory reaction stimulated either by the organism itself, or the underlying diseases. Future studies involving investigation of the effect of *M. pachydermatis* on cytokine production and expression of adhesion molecules by canine keratinocytes would provide further information concerning the regulation of epidermal hyperplasia induced by overgrowth of *Malassezia* organisms.

A single strain of *M. pachydermatis* was used throughout the studies described in this thesis. This was deemed appropriate due to the homogeneity of *M. pachydermatis* that has been demonstrated by different investigators. Senczek *et al.* (1999) compared the electrophoretic karyotypes of *M. pachydermatis* isolated from 198 dogs using pulse-field gel electrophoresis. All isolates of *M. pachydermatis* examined revealed similar electrophoretic karyotypes with the majority showing six bands of chromosomal DNA ranging between 840 and 1850 kBp, regardless of the origin of the isolates (ear/skin/vagina). Coutinho *et al.* (1997) compared the protein profiles of cell wall extracts from *M. pachydermatis* isolated from 15 dogs with otitis

to 15 dogs with dermatitis using SDS-PAGE. The results revealed protein bands ranging from 15 to 146 kDa, however, no statistical differences were found in the protein patterns obtained from the two groups, either with the molecular weights or the number of bands. In a similar study, Bond and Lloyd (2002) obtained *Malassezia* extracts by mechanically disrupting the yeast cells and compared the protein patterns of extracts from 6 different strains of *M. pachydermatis* isolated from dogs with skin diseases or otitis externa using SDS-PAGE. A high level of uniformity was observed between the six strains with each strain showing protein bands with molecular weights of 23-220 kDa, and only minor differences were observed between strains. The protein profiles of culture supernatants from *M. pachydermatis* have also been investigated using the same technique. Although considerable variability was revealed between strains, the protein patterns of concentrated broth culture supernatants from *M. pachydermatis* obtained from healthy dogs did not differ from that of dogs with dermatitis. No clear association between the origin of the strain and the expression of particular proteins was identified (Bond 2002). Furthermore, when used in intradermal testing, the reactivity to two extracts prepared from different strains of *M. pachydermatis* in 8 dogs was shown to be closely correlated, especially at higher concentrations (Bond *et al.* 2002a). Taken together, the results of these studies indicate that antigens of *M. pachydermatis* are likely to be ubiquitous within strains and different strains may have similar antigenicity. Additionally, using a single strain allows more experiments to be performed with limited volumes of serum samples. Due to the low dilution ratio required in the IgE blots (chapter 4), examination of multiple strains would have reduced the number of experiments that could have been performed. Based on all these factors, it was decided to use a single strain of *M. pachydermatis* throughout these studies. However, consideration should

be given to the use of different strains in future studies investigating the interaction between *M. pachydermatis* and canine skin.

In conclusion, this study has contributed to our understanding of the host response to *M. pachydermatis*, and suggested possible mechanisms by which overgrowth of *Malassezia* organisms induces immunological responses and histopathological changes in affected dogs. It is hoped that these findings can be extended in future studies to further define the properties of the 25, 45, 52, 56, and 63 kDa proteins, and to further explore the factors involved in triggering histopathological changes associated with *Malassezia* dermatitis. Studies of protein identification might lead to the development of standardised *M. pachydermatis* allergens for use in intradermal testing, *in vitro* assays and allergen-specific immunotherapy. Also, the recognition of important factors which mediate the formation of epidermal hyperplasia seen in *Malassezia* dermatitis such as cytokines and adhesion molecules might lead to novel approaches to prevent or better control *Malassezia* dermatitis in dogs.

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Publications Arising From This Thesis

1. Chen T. A., Halliwell R. E. W. & Hill P. B. (2002) IgG responses to *Malassezia pachydermatis* antigens in atopic and normal dogs. In: Thoday K. L., Foil C. S. and Bond R. eds. *Advances in Veterinary Dermatology*, Vol. 4. Blackwell Science Ltd, Oxford: pp. 202-209.
2. Chen T. A., Halliwell R. E., Pemberton A. D. & Hill P. B. (2002) Identification of major allergens of *Malassezia pachydermatis* in dogs with atopic dermatitis and *Malassezia* overgrowth. *Veterinary Dermatology* **13**, 141-150.
3. Chen T. A., Halliwell R. E. & Hill P. B. (2002) Failure of extracts from *Malassezia pachydermatis* to stimulate canine keratinocyte proliferation *in vitro*. *Veterinary Dermatology* **13**, 323-329.

Immunoglobulin G responses to *Malassezia pachydermatis* antigens in atopic and normal dogs

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Summary

Overgrowth of *Malassezia pachydermatis* is a common occurrence on the skin of dogs with atopic dermatitis, but the interaction between the organism and the host's immune system has not been extensively investigated. The aim of this study was to characterise one arm of the humoral immune response to *M. pachydermatis* by comparing immunoglobulin G (IgG) responses to antigens separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 18 atopic dogs with *Malassezia* dermatitis, 20 atopic dogs without *Malassezia* dermatitis and 18 clinically normal dogs using Western immunoblotting. Numerous IgG-binding proteins were

detected with sera from atopic dogs with *Malassezia* dermatitis. Strong bands with molecular weights of 25, 29, 42, 45–51, 61, 82 and 90 kDa were seen in more than 50% of the dogs. However, antigens of 28–30, 42, 45–51, 61, 82 and 90 kDa were also recognised by most atopic dogs without *Malassezia* dermatitis. Approximately half of the normal dogs showed faint IgG binding to multiple indistinct bands between 42 and 68 kDa, but clearer bands could be seen at 42, 45 and 82 kDa. These results indicate that the majority of atopic dogs with *Malassezia* dermatitis have a greater IgG response than normal dogs, and show responses to an additional protein of 25 kDa. This antigen may therefore be clinically important in cases of *Malassezia* dermatitis associated with canine atopy.

Introduction

Malassezia pachydermatis is a lipophilic, unipolar-budding yeast that can be isolated from the skin and ear canals of healthy dogs (Baxter, 1976; Gedek *et al.*, 1979; Bond *et al.*, 1994, 1995; Bond & Anthony, 1995; Kennis *et al.*, 1996). However, since the early 1980s, a dermatitis has been reported that is associated with elevated cutaneous populations of *M. pachydermatis* and shows a beneficial response to antifungal therapy (Dufait, 1983; Larsson *et al.*, 1988; Mason & Evans, 1991). The factors that have been suggested to cause overgrowth of the organism are alterations in skin-surface microclimate, such as increased temperature, moisture and sebum production as well as defective host immunity (Mason, 1992). Such changes may be

associated with various underlying skin diseases, including hypersensitivity disorders, bacterial skin infections, keratinisation defects and endocrine diseases (Scott & Miller, 1989; Mason & Evans, 1991; Mason, 1992; Morris, 1999).

Atopic dermatitis is one of the most common diseases associated with *Malassezia* overgrowth in dogs. It has been demonstrated that some atopic dogs carry higher numbers of *M. pachydermatis* on the skin than normal dogs (Bond & Sant, 1993; Bond *et al.*, 1994; White *et al.*, 1996), and cytological evidence of *Malassezia* overgrowth is a common finding in atopic dogs in the authors' clinic (personal observations).

The interaction between *M. pachydermatis* and the host's immune system has not been widely studied either in normal or atopic dogs. Morris *et al.* (1998)

showed that atopic dogs may mount an immunoglobulin E (IgE) response to *M. pachydermatis*, and Nuttall showed that atopic dogs had elevated levels of *Malassezia*-specific IgG (Nuttall, 1997) and IgE (Nuttall, 1998) in their serum.

The aim of this study was to characterise further one arm of the humoral immune response to *M. pachydermatis* by comparing IgG responses to individual antigens of the yeast in atopic dogs with and without *Malassezia* dermatitis and normal dogs, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting.

Materials and methods

Serum samples

Serum samples were collected from 18 atopic dogs with *Malassezia* dermatitis and 20 atopic dogs without *Malassezia* dermatitis presenting to the dermatology clinic at the Royal (Dick) School of Veterinary Studies in Edinburgh. Sera of clinically normal dogs were collected from 18 dogs presenting for euthanasia at a rescue centre.

The diagnosis of atopic dermatitis was based on a combination of consistent history and clinical signs, exclusion of other causes of pruritic skin disease and the presence of at least one positive intradermal skin-test reaction. Coat brushings, skin scrapings and trial therapy were used to eliminate ectoparasites. A 6 week, home-cooked diet trial was conducted to eliminate food intolerance. No anti-inflammatory medication was given for at least 3 weeks before examination. The intradermal skin tests were performed with 57 allergens using 0.05 ml of each allergen extract on the lateral flank after the dogs were sedated with 0.15 mg/kg xylazine intramuscularly (i.m.) and clipped. Histamine (1/100 000 w/v) and saline diluent were also injected as positive and negative controls. Test sites were assessed after 20 min and scored from 0 (negative control) to 4 (positive control) compared with the controls. Reactions ≥ 2 were considered positive (Scott *et al.*, 2001).

Malassezia dermatitis was diagnosed by microscopic observation of Diff-Quik® (Dade AG, Switzerland) stained tape strips (Mason, 1992; Bond & Sant, 1993; Charach, 1997). Samples were obtained from the groin, axilla and interdigital web, and *Malassezia* overgrowth was characterised as an average of five or more *Malassezia* organisms per 400 \times field. *Malassezia*

otitis was diagnosed by sampling the external ear canal with a cotton swab and transferring the material to a glass slide prior to heat fixing and staining. The criteria for demonstrating overgrowth were as described for the tape strips.

Normal dogs had no history or clinical signs of skin disease and had no lesions on dermatological examination.

Culture of *M. pachydermatis*

An isolate of *M. pachydermatis* was obtained from the ear canal of a dog with *Malassezia* otitis. This was deemed appropriate because previous studies have shown that *M. pachydermatis* was the only *Malassezia* species isolated from the skin and external ear canal of dogs with either otitis externa or skin infections, and all isolates of *M. pachydermatis* had similar electrophoretic karyotypes (Senczek *et al.*, 1999). The sample was cultured on Sabouraud Dextrose agar (Oxoid, Basingstoke, UK) containing 20 mg/ml chloramphenicol (Intramycin®; Parke-Davis Veterinary, Eastleigh, UK) for 48 h at 37°C, and the colonies were identified as *M. pachydermatis* by microscopic examination. The colonies were then sub-cultured onto large numbers of plates to obtain enough organisms for subsequent studies.

Extraction of *M. pachydermatis* proteins

Malassezia colonies were carefully harvested and suspended in phosphate-buffered saline (PBS, pH 7.4) for a washing procedure that consisted of three cycles of centrifugation at 500 g for 5 min followed by removal of the supernatant and resuspension in PBS. After the last washing cycle, the cells were resuspended in extraction buffer (pH 7.4) containing 125 mM NH_4HCO_3 (Sigma, Poole, UK) and protease inhibitors (20 mM ϵ -aminocaproic acid, Sigma, UK; 5 mM ethylenediaminetetraacetic acid, Sigma, UK; and 1 mM phenylmethylsulfonyl fluoride, BDH, UK) (Jensen-Jarolim *et al.*, 1992; Morris *et al.*, 1998).

Preliminary experiments were performed using three different methods to determine the optimum method for protein extraction. First, the *Malassezia* colonies in the extraction buffer were mixed vigorously with an equal volume of glass beads (0.4 mm, 40 mesh, BDH, UK) on a vortex mixer for 10 min to disrupt the cell membranes mechanically. A second suspension of *Malassezia* organisms was subjected to three cycles of freezing and thawing by alternately immersing the

container in a dry ice-methanol mixture and warm tap water. This was intended to fracture the cell membranes and allow release of cytoplasmic contents. Thirdly, a *Malassezia* suspension was placed in an ultrasonic water bath for 5 min to fragment the organisms physically. Ten agar plates were prepared for each method, and equal weights of colonies (3.07 g) were added to 5 ml of extraction buffer. After extraction, the cell suspensions were stored at 4°C overnight, then centrifuged at 6000 g for 5 min, and the supernatants were collected. The amount of protein obtained from each method was measured with BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL, USA) and the extracts were analysed by SDS-PAGE. The protein concentrations of the extracts obtained from the three methods (glass beads, freeze-thaw cycles and ultrasonification) were 16.35, 0.58, and 0.22 mg/ml, respectively. Figure 4.6.1 shows the Coomassie blue-stained protein profiles of the three different extracts on an SDS-PAGE gel. Much stronger protein bands were seen in the extract obtained by glass bead

disruption. This method was therefore used in subsequent studies.

SDS-PAGE and immunoblotting

The gel electrophoresis was performed according to the method of Laemmli (1970) using 12% separating polyacrylamide gel and 4% stacking gel in a discontinuous buffer system containing 0.025 M Tris (Sigma, UK), 0.2 M glycine (Fisher Scientific, Loughborough, UK) and 0.1% SDS (Fisher Scientific, UK), pH 8.3. The extracts and molecular weight standards were diluted 1:1 with reducing sample buffer (containing 5% β-mercaptoethanol; Sigma, UK) and heated at 95°C for 5 min. They were then added to the wells of the gel and the electrophoresis was run at 200 V for 60 min.

The separated proteins and molecular weight standards were transferred from the gel to a polyvinylidene difluoride microporous membrane (Millipore Immobilon™-P Transfer Membrane; Millipore Corporation, Bedford, MA, USA) in a BioRad Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell according to

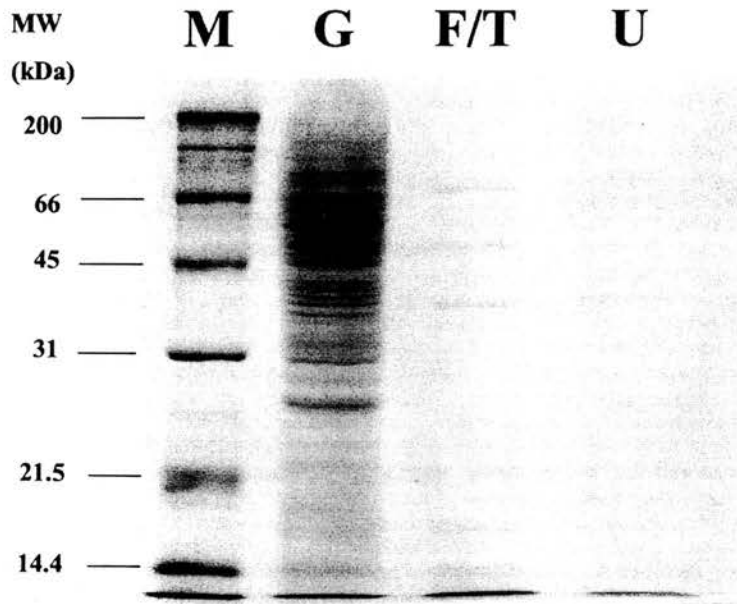


Figure 4.6.1. Extracts of *Malassezia pachydermatis* obtained using three different methods in a Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. M: broad-range molecular weight standard; G: glass beads; F/T: three freeze-thaw cycles; U: ultrasonification. Molecular weights are shown in kilodaltons. The extract produced using glass bead disruption was selected for the immunoglobulin G study.

the manufacturer's instructions. The transfer buffer contained 10 mM (cyclohexylamino)-1-propane sulfonic acid (CAPS, Sigma, Poole, UK) and 10% methanol (Fisher Scientific, UK) (LeGendre *et al.*, 1993). The transfer was run at 80 mA per minigel for 1 h. The quality of transfer was checked by staining gels and molecular weight standards blotted onto the membrane with Coomassie brilliant blue R-250 (BDH, Poole, UK).

For detection of IgG binding proteins, one broad well of *Malassezia* extract (123 µg, 150 µl of 1/20 dilution) running across the top of the gel was used. This allowed the membrane to be cut into narrow strips so that they could be probed with individual sera. They were then blocked for 1 h with 1% skimmed milk in Tris-buffered saline (TBS) containing 0.02 M Tris and 0.5 M NaCl (Sigma, UK), pH 7.5. After washing with TBS containing 0.05% Tween 20 (Fisher Scientific, UK) (TTBS) for 15 min (3 × 5 min rinses), each strip was incubated with 10 µl of dog serum diluted to 1 ml in dilution buffer (1% skimmed milk in TTBS) for 30 min. The strips were then washed again in TTBS for a further 15 min. Bound IgG was detected by incubating the strips for 1 h with horseradish peroxidase-conjugated goat anti-dog IgG heavy and light chain (Bethyl Laboratories, Montgomery, TX, USA) diluted 1:10 000 in dilution buffer. After further washing with TTBS for 15 min, the strips were developed with 3,3'-diaminobenzidine (DAB) peroxidase substrate (Vector Laboratories, Burlingame, CA, USA) for about 1 min. The molecular weights of IgG binding proteins were determined using standard curves generated by

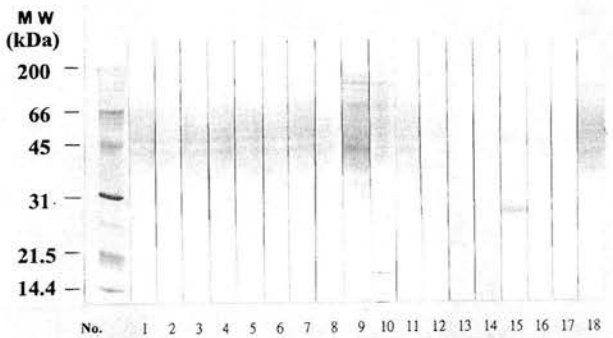
analysing the position of molecular weight markers on Coomassie blue-stained membranes.

Results

The IgG immunoblots of individual dog sera from the three different groups are shown in Figure 4.6.2a–c. The amount of staining seen in the normal dogs (Figure 4.6.2a) was much weaker than that seen in the atopic dogs with cytological evidence of *Malassezia* overgrowth (Figure 4.6.2b). The binding pattern seen in atopic dogs without *Malassezia* dermatitis was between that of the other two groups (Figure 4.6.2c).

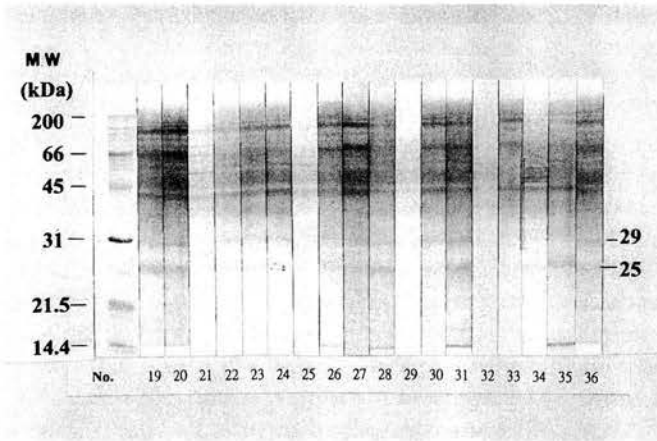
More than half of the normal dogs showed multiple indistinct bands between 42 and 68 kDa, but clearer bands could be seen at 42 (in 83% of dogs), 45 (61%) and 82 kDa (67%) (Figure 4.6.3a). Two dogs showed relatively strong binding patterns (nos 9 and 18) and one dog had a prominent band at 27 kDa (no. 15).

In the atopic dogs with *Malassezia* dermatitis, there was more intense staining of indistinct bands between 45 and 51 kDa. However, distinct IgG binding proteins with molecular weights of 25 (78%), 29 (67%), 42 (94%), 61 (67%), 82 (89%) and 90 kDa (61%) were recognised by more than 50% of the atopic dogs with *Malassezia* dermatitis (Figure 4.6.3b). Some atopic dogs also showed binding to various low molecular weight proteins between 14 and 42 kDa. Antigens of 28–30 (55%), 42 (95%), 45–51 (80%), 61 (50%), 82 (65%) and 90 (55%) kDa were also recognised by more than 50% of the atopic dogs without *Malassezia* dermatitis (Figure 4.6.3c).

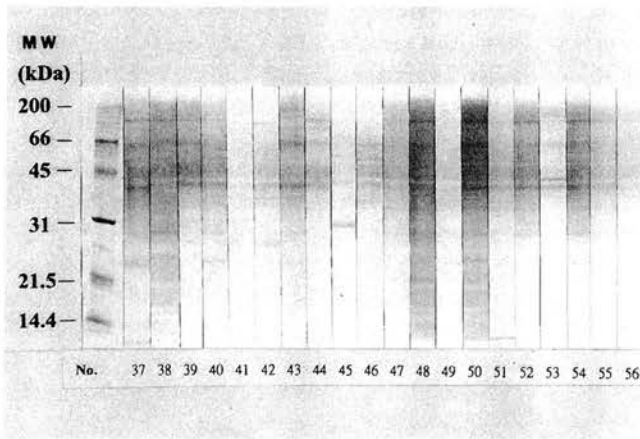


(a)

Figure 4.6.2. Immunoglobulin G-binding components in extracts of *Malassezia pachydermatis* detected by immunoblotting with dog sera from three different groups: (a) normal dogs, (b) atopic dogs with *Malassezia* dermatitis, and (c) atopic dogs without *Malassezia* dermatitis. The numbers along the bottom signify the strips probed with individual dog sera. The molecular weight standards on the left are 200, 66, 45, 31, 21.5 and 14.4 kDa.



(b)



(c)

Figure 4.6.2. (Continued)

The blots of the three groups demonstrated that most dogs showed IgG binding to multiple *Malassezia* antigens. However, atopic dogs with *Malassezia* dermatitis had a much greater IgG response than the normal dogs, and the majority showed binding to a protein of 25 kDa which was not seen in most dogs of the other two groups.

Discussion

The results of the immunoblotting with individual dog sera indicate that most dogs have an IgG response to multiple antigens of *M. pachydermatis*. Approximately half of the normal dogs showed faint IgG binding to multiple indistinct bands between 42 and 68 kDa, but clearer bands could be seen at 42, 45 and 82 kDa. These difficulties in distinguishing bands could be overcome using two-dimensional SDS-PAGE and

immunoblotting (Onishi *et al.*, 1999). Nevertheless, the results suggest that the commensal population of *M. pachydermatis* is recognised by the dog's immune system, leading to an antibody response. Whether or not this IgG response is protective in any way remains to be determined.

The degree and intensity of staining on the blots from the atopic dogs with *Malassezia* dermatitis was much greater than that seen in the normal dogs, indicating that the majority of atopic dogs with *Malassezia* dermatitis had a greater IgG response to *M. pachydermatis*. This result is in accordance with a recent finding that dogs with skin disease associated with *M. pachydermatis* develop high serum titres of *M. pachydermatis*-specific IgG (Bond *et al.*, 1998). Furthermore, more numerous IgG binding proteins were observed on the blots from the atopic dogs with *Malassezia* dermatitis than from the normal dogs. Some of these proteins were the same in

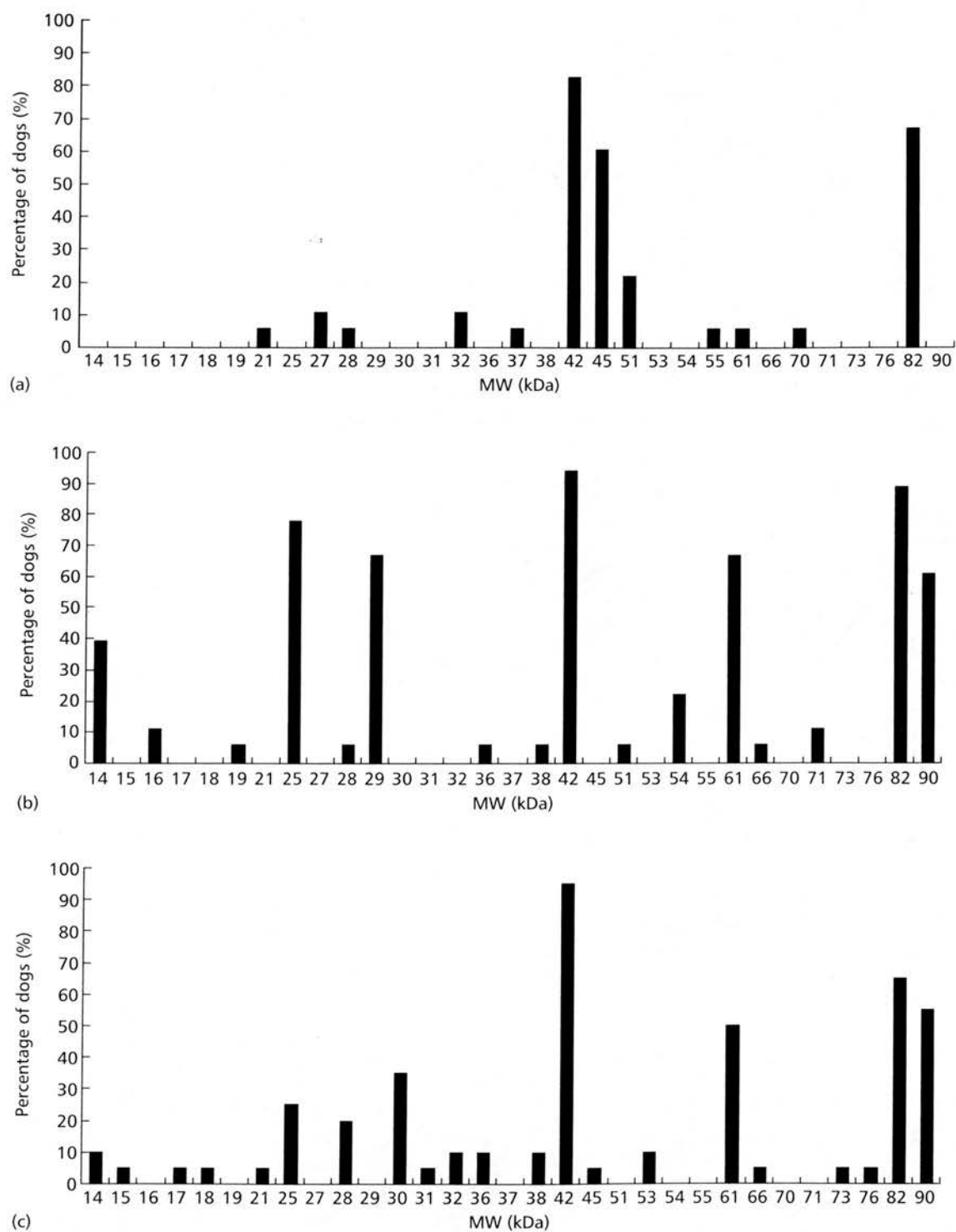


Figure 4.6.3. Percentage of dogs from three different groups showing immunoglobulin G binding to individual antigenic components in extracts of *Malassezia pachydermatis*: (a) normal dogs, (b) atopic dogs with *Malassezia* dermatitis, and (c) atopic dogs without *Malassezia* dermatitis. x-Axis: molecular weights of the bands detected on the strips; y-Axis: percentage of dogs reacting to these bands.

both groups (molecular weights of 42, 45–51 and 82 kDa). However, an antigen of 25 kDa was recognised by the majority of atopic dogs with *Malassezia* dermatitis but was not recognised by most dogs in the other two groups. At this stage, the identity and function of this protein are unknown, as is the significance of the IgG response to this antigen in the atopic dogs. The IgG may act as an opsonin to facilitate phagocytosis of *Malassezia* antigens. This could have a protective function but it could also activate the complement system and exacerbate the underlying inflammation. It has been shown that *Malassezia furfur* is capable of activating both the classical and alternative complement pathways (Belew *et al.*, 1980; Sohnle & Collins-Lech, 1983). It has also been suggested that zymogen in the cell wall of *M. pachydermatis* might activate complement, leading to epidermal damage and inflammation (Mason, 1993). Despite the uncertainty surrounding the role of this antigen, the IgG response seen in the atopic dogs with *Malassezia* overgrowth but not in the normal dogs suggests that it may have some clinical relevance in the disease.

IgG binding components in extracts of *M. pachydermatis* have also been investigated with sera of healthy dogs and non-atopic dogs with *Malassezia* dermatitis (Bond & Lloyd, 1999). Proteins of 132, 66 and 50–54 kDa were recognised by most sera, but affected dogs showed immunoreactivity mainly to proteins of 219, 110, 71 and 42 kDa (Bond & Lloyd, 1999). Comparing the relative positions of bands and molecular weight standards, it may be inferred that some of these results are likely to represent the same bands as those seen in the present study. However, the 25 kDa antigen was not recognised in the non-atopic dogs with *Malassezia* dermatitis, again suggesting a degree of specificity for this response in dogs with atopic dermatitis.

The pattern of IgG binding seen in the atopic dogs without *Malassezia* dermatitis was between that of the normal dogs and atopic dogs with *Malassezia* dermatitis. Most of these dogs also recognised antigens of 28–30, 42, 45–51, 61, 82 and 90 kDa. Owing to the difficulty in precisely resolving the molecular weight of proteins on SDS–PAGE gels, it is possible that the bands detected at 28 and 30 kDa are the same as the 29 kDa antigen seen in the group with *Malassezia* overgrowth. There are some possible explanations for this intermediate pattern. White *et al.* (1996) compared the carriage of *M. pachydermatis* on clinically normal skin of the intradermal skin-test area in atopic dogs and

trunkal glabrous skin in healthy dogs. They found that atopic dogs had significantly higher yeast culture scores than healthy dogs. Hence, the atopic dogs without *Malassezia* dermatitis could still have had elevated populations of the yeast without having cytological evidence of an overgrowth. In addition, some of the atopic dogs in the present study may have had *Malassezia* dermatitis in the past, leading to a residual IgG response. Finally, the immune system of atopic dogs may be sensitized to the normal population of the commensal through their frequent scratching behaviour. It has been suggested that disruption of stratum corneum barrier function by scratching may enhance exposure of the skin immune system to yeast allergens (Morris *et al.*, 1998). However, the 25 kDa antigen was only seen in less than 25% of the atopic dogs without *Malassezia* overgrowth, suggesting that an IgG response to this protein is clinically relevant in the disease.

In human beings, *M. furfur* is believed to be significant in the pathogenesis of atopic dermatitis, and numerous IgE binding proteins have been identified in crude extracts using immunoblotting techniques (Johansson & Karlstrom, 1991; Jensen-Jarolim *et al.*, 1992; Zargari *et al.*, 1994; Lintu *et al.*, 1997). Furthermore, type I hypersensitivity responses towards intradermally injected extracts of *M. pachydermatis* have been reported in atopic dogs with *Malassezia* dermatitis (Morris *et al.*, 1998). Hence, to complement the IgG data presented in this paper, and to elucidate further the role of *M. pachydermatis* in atopic dermatitis, studies are currently underway to define the IgE response to *Malassezia* antigens in atopic dogs.

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Identification of major allergens of *Malassezia pachydermatis* in dogs with atopic dermatitis and *Malassezia* overgrowth

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Abstract We have previously shown that both atopic and normal dogs generate an IgG response to antigens of *Malassezia pachydermatis*. The aim of this study was to compare IgE responses to separated proteins of *M. pachydermatis* in 28 atopic dogs with *Malassezia* dermatitis and 22 clinically normal dogs using Western immunoblotting. Six different detection systems were evaluated in order to assess sensitivity and eliminate non-specific binding and cross-reactivity. The protocol yielding the best results utilized a monoclonal mouse antidog IgE, an alkaline phosphatase conjugated goat antimouse IgG which had been passed through a canine IgG column 3 times, a chemiluminescent substrate and a digital imaging system. Proteins of 45, 52, 56 and 63 kDa were recognized by more than 50% of the atopic dog sera and thus represented major allergens. Only a minority of normal dogs showed faint IgE binding to these proteins. The results indicate that the majority of atopic dogs with *Malassezia* dermatitis have a greater IgE response than normal dogs, suggesting an IgE-mediated immune response may be clinically important in the pathogenesis of the disease.

Keywords: atopic dermatitis, dogs, IgE, *Malassezia*

INTRODUCTION

Malassezia pachydermatis, a lipophilic, unipolar-budding yeast is a commensal on canine skin and it can be isolated from the skin and ear canals of healthy dogs.^{1–6} However, a dermatitis associated with elevated cutaneous populations of *M. pachydermatis* that shows a beneficial response to antifungal therapy has been reported in the last two decades,^{7–9} indicating that the organism is an opportunistic pathogen. Several factors have been suggested to cause overgrowth of the organism, such as alterations in skin surface microclimate and defective host immunity.¹⁰ These factors may be associated with various underlying skin diseases including hypersensitivity disorders, bacterial skin infections, keratinization defects and endocrine diseases.^{9–12}

Atopic dermatitis is one of the most common diseases associated with *Malassezia* overgrowth in dogs, and it has been demonstrated that some atopic dogs carry higher numbers of *M. pachydermatis* on their non-lesional and lesional skin than normal dogs.^{3,13,14} Immediate hypersensitivity responses to intradermal injections of *M. pachydermatis* extracts have been demonstrated in atopic dogs with *Malassezia* overgrowth,¹⁵ suggesting that allergens from the organism may be involved in the pathogenesis of atopic dermatitis.

Previous reports have also shown that atopic dogs have elevated levels of *Malassezia*-specific IgG and IgE¹⁶ in their serum.

In human patients with atopic dermatitis, *Malassezia furfur* is known to be allergenic, and a number of IgE-binding components in the range of 9–100 kDa have been documented.^{17–20} Nine allergens of *Malassezia furfur* (Mal f 1–Mal f 9) have now been sequenced and expressed as recombinant proteins,^{21–25} with molecular weights of 37, 21, 20, 35, 18.2, 17.2, 16.2, 19.2 and 14 kDa, respectively.

We have recently shown that the majority of atopic dogs with *Malassezia* dermatitis have a greater IgG response to the yeast than normal dogs, and that an antigen of 25 kDa appears to be clinically important.²⁶ The aim of this study was to further characterize the humoral immune response to *M. pachydermatis* by comparing IgE responses to separated antigens of the yeast in atopic dogs with *Malassezia* dermatitis and normal dogs.

MATERIALS AND METHODS

Serum samples

Serum samples were collected from 28 atopic dogs with *Malassezia* dermatitis presented to the dermatology clinic at the Royal (Dick) School of Veterinary Studies in Edinburgh. Sera of clinically normal dogs were collected from 22 dogs presented for euthanasia at a rescue centre.

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The diagnosis of atopic dermatitis was based on a combination of consistent history and clinical signs, exclusion of other causes of pruritic skin disease and the presence of at least one positive intradermal skin test reaction. Coat brushings, skin scrapings and trial therapy were used to rule out ectoparasites. A 6-week, home-cooked diet trial was conducted to rule out adverse food reactions. No anti-inflammatory medication was given for at least 3 weeks prior to examination. The intradermal skin tests were performed with 57 allergens using 0.05 mL of each allergen extract on the lateral flank after the dogs were sedated with 0.15 mg kg⁻¹ xylazine intramuscularly and clipped. Histamine (1/100 000 w/v) and diluent consisting of buffered saline with 0.4% phenol as preservative were also injected as positive and negative controls, respectively. Test sites were assessed after 20 min and scored from 0 to +4 by comparison with the controls. Reactions ≥ 2 were considered positive.²⁷

Malassezia overgrowth was diagnosed by microscopic observation of Diff-Quik® (Dade AG, Switzerland) stained tape strips.^{10,13,28} Samples were obtained from the groin, axilla and interdigital web and *Malassezia* overgrowth was characterized as an average of 5 or more *Malassezia* organisms per 400 \times field.^{29,30} Involvement of *Malassezia* in cases of otitis externa was assessed by sampling the external ear canal with a cotton swab and transferring the material to a glass slide prior to heat fixing and staining. The criteria for demonstrating overgrowth were as described for the tape strips.

Normal dogs had no history or clinical signs of skin disease and had no lesions on dermatological examination.

Culture of *M. pachydermatis*

An isolate of *M. pachydermatis* was obtained from the ear canal of a dog with *Malassezia* otitis. The use of a single isolate was deemed appropriate because previous studies have shown that *M. pachydermatis* was the only *Malassezia* species isolated from the skin and external ear canal of dogs with either otitis externa or skin infections, and all isolates of *M. pachydermatis* had similar electrophoretic karyotypes.³¹ The sample was cultured on Sabouraud Dextrose agar (Oxoid, UK) containing 20 mg mL⁻¹ chloramphenicol (Intramy-cetin®, Parke-Davis Veterinary, UK) for 48 h at 37 °C, and the colonies were identified as *M. pachydermatis* by microscopic examination. The colonies were then subcultured onto large numbers of plates in order to obtain enough organisms for subsequent studies.

Extraction of *M. pachydermatis* proteins

Malassezia colonies were carefully harvested and suspended in phosphate-buffered saline (PBS) (pH 7.4) for a washing procedure that consisted of 3 cycles of centrifugation at 500 g for 5 min followed by removal of the supernatant and resuspension in PBS. After the last washing cycle, the cells were resuspended in extraction buffer (pH 7.4) containing 125 mM NH₄HCO₃ (Sigma,

UK) and protease inhibitors (20 mM ϵ -aminocaproic acid, Sigma, UK; 5 mM ethylenediaminetetra-acetic acid, Sigma, UK; and 1 mM phenylmethylsulphonyl fluoride, BDH, UK).^{15,18} The *Malassezia* colonies in the extraction buffer were then mixed vigorously with an equal volume of glass beads (0.4 mm, 40 mesh, BDH, UK) on a vortex mixer for 10 min to mechanically disrupt the cell membranes. After extraction, the cell suspensions were stored at 4 °C overnight, centrifuged at 6000 g for 5 min and the supernatants were collected. The amount of protein obtained was measured with BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL, USA).

SDS-PAGE and electrophoretic transfer

Gel electrophoresis was performed according to the method of Laemmli³² using 10% separating polyacrylamide gel and 4% stacking gel in a discontinuous buffer system containing 0.025 M Tris (Sigma, UK), 0.2 M Glycine (Fisher Scientific, UK) and 0.1% sodium dodecyl sulphate (Fisher Scientific, UK), pH 8.3. The extract (60 μ g) and molecular weight standards were diluted 1:1 with reducing sample buffer (containing 5% β -Mercaptoethanol, Sigma, UK) and heated at 95 °C for 5 min. The extract was then added to one broad well across the top of the gel and the electrophoresis was run at 200 V for 40 min. Fig. 1 shows the protein profile of *M. pachydermatis* extracts on a 10% separating polyacrylamide gel.

The separated proteins and molecular weight standards were transferred from the gel to a polyvinylidene difluoride microporous membrane (Millipore Immobilon™-P Transfer Membrane, Millipore Corporation, Bedford, MA, USA) in a Bio-Rad Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell according to the manufacturer's instructions. The transfer buffer contained 25 mM Tris (Sigma, UK), 192 mM Glycine (Fisher Scientific, UK) and 20% methanol (Fisher Scientific, UK), pH 8.3. The transfer was run at 80 mA per minigel for 1 h. The quality of transfer was checked by staining gels and molecular weight standards blotted onto the membrane with Coomassie Brilliant Blue R-250 (BDH, UK).

Optimization of methodology to detect IgE binding proteins on the *Malassezia pachydermatis* immunoblots

Various methodologies and reagents were evaluated to detect IgE binding proteins in *M. pachydermatis* (summarized in Table 1). Two methods were investigated to allow the immunoblot membrane to be probed with individual dog sera: cutting the membrane into narrow strips or placing the whole membrane into a multichannel miniblitter (Immunitics Inc., Cambridge, MA, USA). The blocking reagents that were evaluated included 5% skimmed milk, casein solution (Vector Laboratories Inc., Burlingame, CA, USA), Tween 20 (Fisher Scientific, UK), Tween 80 (Sigma, UK), and an avidin/biotin blocking kit (Vector Laboratories Inc., Burlingame, CA, USA). The following IgE-specific reagents were evaluated: a polyclonal epsilon chain

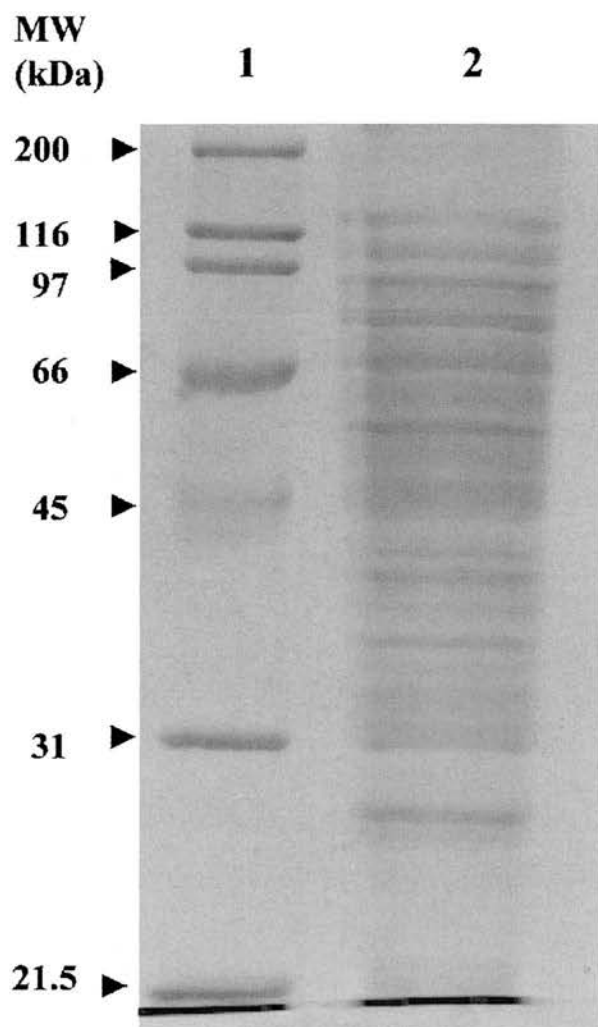


Figure 1. Coomassie Brilliant Blue stained *M. pachydermatis* extracts on a 10% separating polyacrylamide gel. Lane 1: molecular weight markers; lane 2: *Malassezia* extract.

specific goat antidog IgE conjugated with horseradish peroxidase (HRP) (Bethyl Laboratories, Montgomery TX, USA); two monoclonal mouse antidog IgE antibodies (D9#3116, kindly donated by Dr D. J. Deboer, University of Wisconsin, USA and E6-71A1, Custom Monoclonals International, W. Sacramento, CA, USA); and a biotinylated recombinant human IgE receptor alpha chain (kindly donated by Dr C. McCall, Heska Corporation, Fort Collins, USA). For visualization of binding, the following conjugates labelled with either alkaline phosphatase (AP) or HRP were used: a monoclonal mouse antigoat IgG-AP (Sigma, UK); a monoclonal bovine antimouse IgG1-HRP (Serotec, UK); two polyclonal goat antimouse IgG antibodies labelled either with HRP (Sigma, UK) or AP (Southern Biotechnology Associates Inc., USA); and two avidin reagents (Vectastain® Elite ABC kit, Vector Laboratories Inc., Burlingame, CA, USA and Extravidin®-peroxidase, Sigma, UK). Two different detection systems were evaluated: substrate colour change and chemiluminescence. For standard blots, DAB (3,3'-diaminobenzidin, Vector Laboratories Inc., Burlingame,

CA, USA) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma, UK) were used to detect HRP- and AP-labelled reagents, respectively. For chemiluminescent blots, ECL™ (Amersham Pharmacia Biotech, UK) was used for HRP-labelled reagents and CDP-Star™ (Tropix Inc., Bedford, USA) for AP-labelled reagents (Table 1).

Assessment of reagent specificity

Purified dog IgG (Sigma, UK), dog IgE (Bethyl Laboratories, Montgomery TX, USA) and mouse IgG1κ (Sigma, UK) were used to test the specificity of reagents. The purified antibodies were diluted 1:1 with nonreducing sample buffer (in view of the sensitivity of dog IgE to 2-mercaptoethanol³³), and the gel electrophoresis performed as described above. They were then transferred from the gel to a membrane, blocked with 5% skimmed milk in Tris-buffered saline containing 0.02 M Tris, 0.5 M NaCl and 0.1% Tween 20 (TTBS), pH 7.5 for 1 h and sequentially incubated with the various combinations of IgE-specific reagents and secondary antibody conjugates summarized in Table 1.

The antibody conjugate used in protocol 6 had previously been shown to exhibit cross-reactivity between mouse IgG1 and canine IgG (data not shown). To eliminate this cross-reactivity, the conjugate had been further purified using affinity chromatography. Briefly, purified dog IgG (10 mg) dissolved in 0.15 M NaCl (Sigma, UK) was exchanged with sodium hydrogen carbonate buffer (0.2 M NaHCO₃ and 0.5 M NaCl, pH 8.5) using HiTrap Desalting (Amersham Pharmacia Biotech, UK) and coupled to cyanogen bromide (CNBr)-activated Sepharose™ 4B (Amersham Pharmacia Biotech, UK). The remaining active sites in the Sepharose were blocked with 1 M ethanolamine (Sigma, UK), and the gel was then packed into a column. The AP-labelled goat antimouse IgG (Southern Biotechnology Associates Inc., USA) was then passed through the canine IgG column on three occasions using an FPLC® system (Amersham Pharmacia Biotech, UK). Antibodies that did not bind to canine IgG were collected. The specificity of solution passed through the column was checked after each run using the method described above. After three passages through the column, the AP-labelled goat antimouse IgG did not bind to either dog IgE without the presence of monoclonal mouse antidog IgE or to dog IgG with or without the presence of antidog IgE antibody (Fig. 2). For long-term storage, the final solution collected was concentrated using Centriplus™ concentrators (Amicon Inc., Beverly, USA) and the antibody concentration was measured with a spectrophotometer. The concentrated solution was diluted 1:1 in glycerol (BDH, UK) to a final concentration of 0.29 mg mL⁻¹.

Immunoblotting

Evaluation of the various reagents and protocols revealed that an immunoblot membrane cut into strips and protocol 6 (Table 1) yielded the best result. The miniblitter did not produce repeatable results, and

Table 1. Methodologies and reagents evaluated in this study to detect IgE binding proteins in *M. pachydermatis*

Protocol	IgE-specific Reagent	Secondary developing reagent	Substrate	Detection	Comments
1	Goat antidog IgE-epsilon chain specific, HRP (Bethyl Laboratories)		DAB	Colour change on blots	Abandoned due to lack of sensitivity
2	Goat antidog IgE-epsilon chain specific, HRP (Bethyl Laboratories)	Monoclonal anti goat IgG, AP (Sigma)	BCIP/NBT	Colour change on blots	Abandoned due to lack of sensitivity
3	Monoclonal mouse antidog IgE (Dr D.J. Deboer)	Goat antimouse IgG, HRP (Sigma)	ECL™	Chemiluminescence detected on X-ray films (Hyperfilm™ ECL™, Amersham Life Science)	Abandoned due to cross-reactivity of the secondary antibody with canine IgG
4	Monoclonal mouse antidog IgE (Dr D.J. Deboer)	Bovine antimouse IgG1, HRP (Serotec)	ECL™	Chemiluminescence detected on X-ray films	Abandoned due to cross-reactivity of the secondary antibody with canine IgG
5	Biotinylated recombinant human IgE receptor alpha chain (Heska)	Vectastain® Elite ABC kit (Vector Laboratories) Extravidin®-peroxidase (Sigma)	ECL™	Chemiluminescence detected on X-ray films	Abandoned due to cross-reactivity of the secondary-developing reagents with <i>Malassezia</i> extracts
6	Monoclonal mouse antidog IgE E6-71A1 (Custom Monoclonals International)	Goat antimouse IgG1, AP (Southern Biotechnology Associates) before and after passing through a canine IgG column three times	CDP-Star™	Chemiluminescence detected using Kodak Digital Science™ Image Station 440CF	Successful

HRP: horseradish peroxidase; AP: alkaline phosphatase; DAB: 3,3'-diaminobenzidine; BCIP/NBT: 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

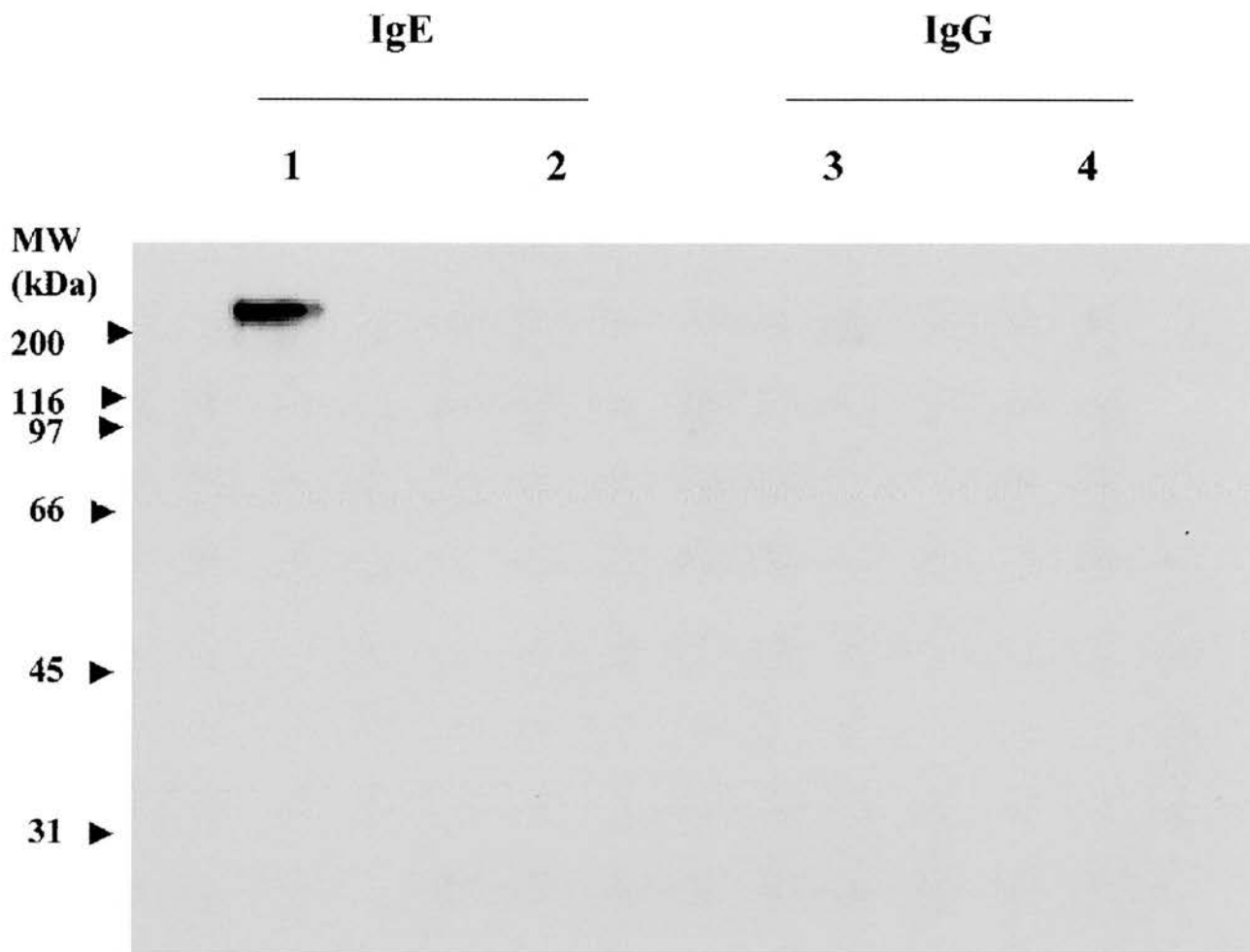


Figure 2. Examination of reagent specificity in protocol 6 to detect IgE-binding proteins in *Malassezia pachydermatis*. Purified dog IgE (lanes 1 and 2) and IgG (lanes 3 and 4) were diluted with nonreducing sample buffer, separated in 10% SDS-PAGE (0.2 µg per lane), and transferred to a membrane. The membranes were then sequentially incubated with monoclonal mouse antidog IgE (E6-71A1) diluted 1:500 in dilution buffer (1% skimmed milk in TTBS), the secondary developing reagent which had been passed through a canine IgG column three times (1:2000 dilution) and the substrate (lanes 1 and 3). For the control groups (lanes 2 and 4), the antidog IgE antibody was replaced with the dilution buffer.

protocols 1–5 were rejected due to lack of sensitivity or specificity. The individual serum samples were therefore analysed using the following conditions. The strips were blocked for 1 h with 5% skimmed milk in TTBS. After washing with TTBS for 15 min (3 × 5 minute rinses), each strip was incubated with individual dog serum diluted 1:5 in dilution buffer (1% skimmed milk in TTBS) for 1 h. The strips were then washed again in TTBS for a further 15 min. Bound IgE was detected by incubating the strips for 1 h with monoclonal mouse antidog IgE E6-71A1 (diluted 1:500). After further washing with TTBS for 15 min, the strips were incubated with AP-labelled goat antimouse IgG1 (diluted 1:2000) for 1 h. The strips were washed again with TTBS for 15 min, and then rinsed with detection buffer containing 0.1 M Tris-HCl, 0.1 M NaCl and 5 mM MgCl₂ (BDH, UK), pH 9.5. The strips were developed by incubating them with a chemiluminescent substrate, CDP-StarTM, for 5 min. After draining off excess liquid, the strips were wrapped in cling film and placed on a Kodak Digital ScienceTM Image Station 440CF along with Coomassie blue stained molecular weight

markers. The membrane was exposed for 15 min, and blots were visualized on the computer screen. Kodak Digital ScienceTM 1D Image Analysis Software was used to detect the bands on the blots and to determine the molecular weight of bands by analysing their positions relative to molecular weight markers.

To confirm that the major bands on the blots represented IgE-binding proteins, a pooled serum sample was generated from all the atopic dogs and from all the normal dogs. The pooled samples were analysed by SDS-PAGE and immunoblotting as described above both before and after heating to 56 °C for 2, 4 and 8 h.

RESULTS

Immunoblotting with individual dog sera

The IgE immunoblots of individual dog sera from the two groups are shown in Fig. 3(a and b). The intensity of binding seen in normal dogs (Fig. 3a) was much weaker than that seen in the atopic dogs with *Malassezia* dermatitis (Fig. 3b).

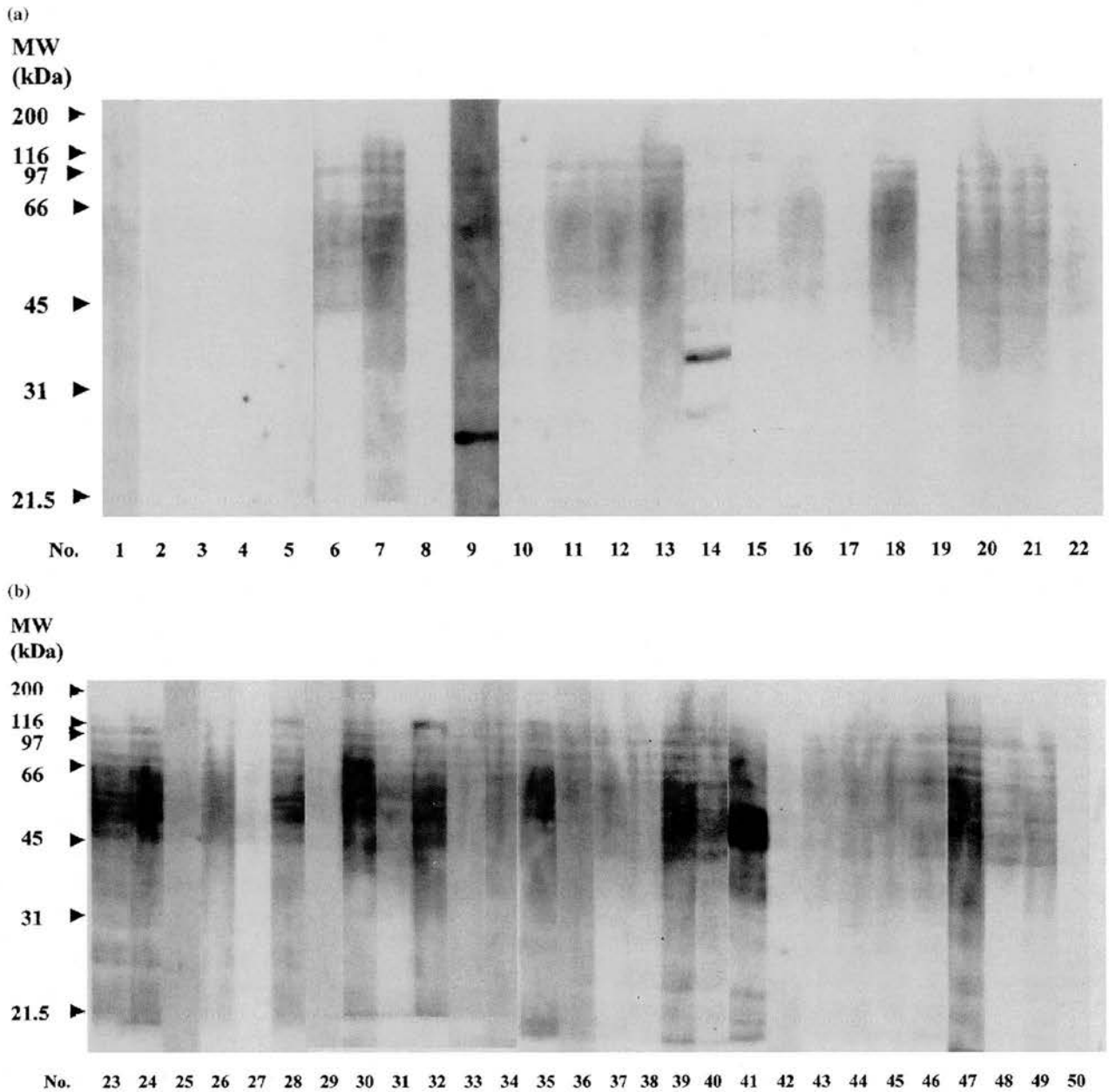


Figure 3. IgE-binding proteins in extracts of *M. pachydermatis* detected by immunoblotting with dog sera from 2 groups: (a) normal dogs; and (b) atopic dogs with *Malassezia* dermatitis. The images of blots were captured by a digital imaging station and printed out directly from the computer. The numbers along the bottom signify the strips probed with individual dog sera. The molecular weight markers on the left are 200, 116, 97, 66, 45, 31 and 21.5 kDa.

The intensity of binding in atopic dogs with *Malassezia* dermatitis was strongest between 45 and 63 kDa. IgE binding proteins with molecular weights of 45 (61% of dogs), 52 (50%), 56 (61%) and 63 (68%) kDa were recognized in more than 50% of the dogs (Fig. 4). Strong bands were also detected at 26 (29%), 48 (39%) and 60 (18%) kDa.

Approximately one-third of the normal dogs showed faint binding to proteins between 45 and 63 kDa, but clearer bands at 45 (in 18% of dogs), 52 (36%), 56 (32%) and 63 kDa (36%) were detected by the image analysis software (Fig. 4). Furthermore, one dog had a prominent band at 26 kDa (No. 9) (Fig. 3a).

In order to confirm that the bands detected on the blots were IgE-binding proteins, a pooled serum sample was prepared from the atopic dogs with

Malassezia dermatitis and from the normal dogs. The pooled serum samples were heat treated at 56 °C for 2, 4 and 8 h to detect the heat lability of binding reactions (Fig. 5). The binding patterns seen in the two serum pools were very similar to those seen in the corresponding individual serum samples. As with the individual blots, the binding intensity seen in the atopic dog serum pool was much stronger than that in the normal dogs. Between 45 and 63 kDa, the binding intensity declined dramatically over the period when serum pools were heated. Gradually decreasing binding activity was also seen on one single band at 26 kDa. Accordingly, bands of 26 kDa and any binding detected between 45 and 63 kDa on the strips from the individual dogs were regarded as IgE specific.

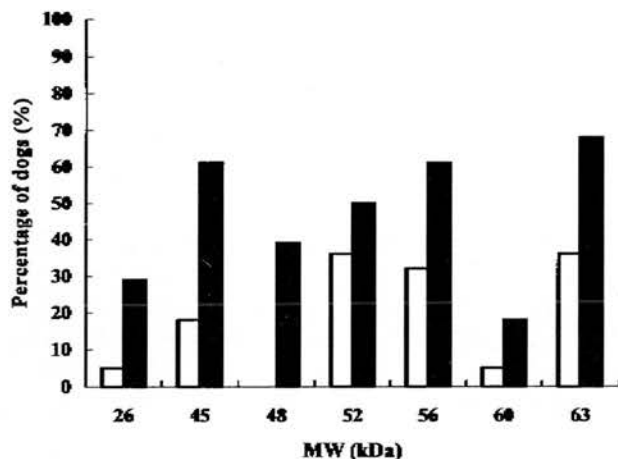


Figure 4. The percentage of dogs from the two groups showing IgE binding to individual proteins in extracts of *M. pachydermatis*. The bands on the blots shown in Fig. 3 were detected using Kodak Digital Science™ 1D Image Analysis Software and the molecular weight of bands generated by the software was used to calculate the percentage of dogs reacting to individual bands. Filled columns: atopic dogs with *Malassezia* dermatitis; open columns: normal dogs. x-axis: molecular weights of the bands detected on the strips; y-axis: percentage of dogs reacting to those bands.

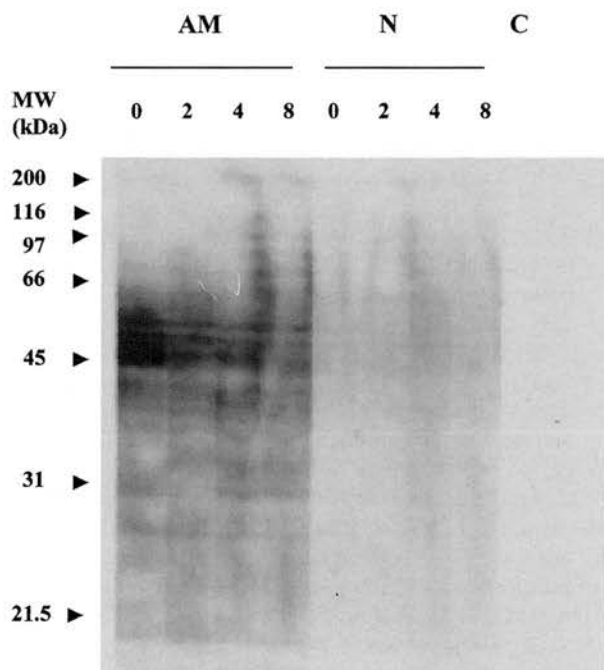


Figure 5. Heat-lability of binding detected in two serum pools: one from atopic dogs with *Malassezia* dermatitis and one from normal dogs. AM: atopic dogs with *Malassezia* dermatitis; N: normal dogs; C: control (dilution buffer: 1% skimmed milk in TTBS). The numbers represent the length of heat treatment. 0: nonheated serum pool; 2, 4, and 8: after heating for 2, 4 and 8 h, respectively. Note the decrease in staining intensity seen in bands at 26 and 45–63 kDa after heating.

DISCUSSION

In this study, we have shown that *Malassezia* dermatitis in atopic dogs is associated with an IgE response to the organism (compare Fig. 3a and b). This result con-

firms the findings of previous studies that have demonstrated elevated concentrations of *Malassezia*-specific IgE in atopic dogs with *Malassezia* overgrowth by ELISA¹⁶ and positive responses following intradermal injection of *Malassezia* extracts.¹⁵ Furthermore, we have demonstrated that the IgE response is predominantly targeted against proteins with molecular weights of 45, 52, 56 and 63 kDa. As these proteins were recognized in over 50% of the atopic dogs with *Malassezia* dermatitis, but only recognized by a minority of normal dog sera, they can be classified as major allergens. Strong bands were also detected with molecular weights of 26, 48 and 60 kDa, but as these were recognized in less than half of the atopic dogs they can be regarded as minor allergens. Weak IgE binding was also seen in some normal dogs between 45 and 63 kDa. The cause of this weak IgE binding in normal dogs is unknown. It has been shown that extracts of *M. pachydermatis* at high concentrations are capable of inducing wheal and flare responses in normal dogs.¹⁵ This could be an irritant reaction or an IgE response present to commensal populations of the yeast. The latter is certainly the case with IgG responses as shown in previous studies.^{16,26}

Of all the allergens detected, those with molecular weights of 45 and 48 kDa seemed to be the most relevant because they were recognized by most atopic dogs with *Malassezia* overgrowth but only a few normal dogs (Fig. 4). Further studies are required to determine the identity of these proteins using protein purification and N-terminal sequencing. They may represent two different proteins or a single protein with variable glycosylation.

In contrast to the low molecular weight allergens of *Malassezia furfur* (Mal f 1–Mal f 9) recognized by humans, dogs recognized higher molecular weight allergens in this study. Similar findings have been documented with *Dermatophagoides* allergens. Most human atopic sera have high IgE titres to group 1 and group 2 allergens of *Dermatophagoides*^{34,35} which are low molecular weight proteins, whereas in dogs the most important *Dermatophagoides* allergens are high molecular weight proteins greater than 90 kDa.^{36–38}

Whilst optimizing the methodology to detect IgE binding proteins in *M. pachydermatis*, we had to overcome problems of lack of sensitivity and specificity (summarized in Table 1). Protocols 1 and 2 were rejected because there were no visible bands, even with the amplification step introduced in protocol 2. To increase the sensitivity, various chemiluminescent protocols were investigated (protocols 3–6). Luminescent detection methods are believed to provide several advantages over traditional chromogenic procedures, such as generally increasing the sensitivity of both HRP and AP systems, rapid detection and long-lasting signals permitting multiple exposures.³⁹ To increase the specificity of the chemiluminescent methods, monoclonal antibodies and an IgE receptor reagent were used. In protocols 3 and 4, the secondary antibodies cross-reacted with canine IgG and they were

abandoned due to lack of specificity. This problem is confounded by the fact that levels of allergen-reactive IgG may be several thousandfold higher than levels of IgE in normal and atopic animals.⁴⁰ The results of our previous study obtained using a less sensitive substrate (DAB) to detect *Malassezia*-specific IgG verified that the levels of antigen-specific IgG were much higher than levels of IgE. The high levels of *Malassezia*-specific IgG in dog sera could enhance the IgG signals and eclipse the IgE reaction.

The high-affinity receptor for IgE, FcεRI, is a requisite for the IgE-mediated immediate hypersensitivity reaction and the interaction between IgE and FcεRI is highly specific.⁴¹ In spite of offering absolute specificity for IgE, the FcεRI receptor reagent used in protocol 5 was not successful because the avidin reagents bound to *Malassezia* proteins. This could stem from endogenous protein-bound biotin or lectins in the extracts, as these contained a crude mixture of both cytoplasmic and cell wall components.

In protocol 6, a combination of reagents that had previously been used successfully to detect IgE binding in ELISA assays (unpublished observations) was assessed. In this protocol, the cross-reactivity of the secondary antibody was removed by immunoabsorption through a canine IgG column. Although protocol 6 proved to be successful, it is possible that the use of the cross-absorbed secondary reagent with other primary antibodies could also be suitable. With this protocol, we also used a digital imager to resolve the bands, rather than assessment of X-ray films. We found that X-ray films produced previously reported problems such as weak bands being overshadowed by adjacent strong signals, broad diffuse images resulting from overexposure and film saturation. We found that the image station and image analysis software provided better resolution of the bands detected and enabled more accurate determination of their molecular weights than traditional chemiluminescent methods. It is important to note that chemiluminescent blots are not visible to the naked eye and can only be visualized on X-ray film or on a computer screen (when using digital imaging). Furthermore, the bands on chemiluminescent blots are not as clear and distinct as those seen on normal chromogenic blots. However, the use of imaging software allows the bands to be resolved without investigator bias.

Despite the various problems associated with lack of specificity, we believe that there are three lines of evidence that confirm that we have detected *Malassezia*-specific IgE. First, using protocol 6 we demonstrated that the reagents did not cross-react with canine IgG (see Fig. 2). Secondly, the IgE binding to the major allergens was shown to be heat labile (Fig. 5). Thirdly, the antigens detected in this study were different from those detected in our previous study using reagents to detect IgG.

In summary, we have demonstrated that *M. pachydermatis* allergens of 45, 52, 56 and 63 kDa appear to be clinically relevant in atopic dogs. However, further

characterization of these allergens is needed before they could potentially be used in intradermal testing or allergen immunotherapy in affected dogs.

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Résumé Nous avons démontré précédemment que les chiens atopiques comme les chiens normaux synthétisaient des IgG en réponse à un stimulus par des antigènes de *Malassezia pachydermatis*. Le but de cette étude était de comparer la synthèse d'IgE vis à vis de différentes protéines de *M. pachydermatis* chez 28 chiens atopiques présentant une dermatite à *Malassezia* et chez 22 chiens cliniquement sains. Six systèmes différents de détection ont été évalués afin d'améliorer la sensibilité et d'éliminer les réactions non spécifiques ou les réactivités croisées. Le protocole donnant les meilleurs résultats était celui avec un anti-IgE monoclonal de souris, un système de phosphatases alcalines conjuguées avec des IgG antisouris de chèvre, qui ont été passées trois fois sur une colonne d'IgG canines, un substrat de chimioluminescence et un système d'imagerie digitale. Des protéines de 45, 52, 56 et 63 kDa ont été reconnues par plus de 50% des sérums de chiens atopiques, et doivent donc considérées comme des allergènes majeurs. Seule une minorité des chiens normaux ont présenté une réactivité IgE faible à ces protéines. Les résultats de cette étude indiquent que la majorité des chiens souffrant de dermatite à *Malassezia* ont une réponse IgE plus élevée que les chiens normaux, suggérant que la réponse immunitaire médiée par les IgE soit importante cliniquement dans la pathogénie de la maladie.

Resumen En estudios anteriores hemos demostrado que tanto perros atópicos como normales generan una respuesta de IgG a antígenos de *Malassezia pachydermatis*. El objetivo de este estudio fue comparar las respuestas de IgE a proteínas separadas de *M. pachydermatis* en 28 perros atópicos con dermatitis por *Malassezia* y 22 perros clínicamente normales, utilizando Western immunoblotting. Se evaluaron seis sistemas diferentes de detección con el fin de evaluar la sensibilidad y eliminar las uniones inespecíficas y la reactividad cruzada. El protocolo con los mejores resultados utilizaba una IgE monoclonal de ratón anti-canina, una IgG caprina anti-murina conjugada con fosfatasa alcalina pasada por una columna de IgG canina 3 veces, un substrato quimioluminescente y un sistema de imagen digital. Las proteínas de 45, 52, 56 y 63 kDa fueron reconocidas por más del 50% de sueros de perros atópicos, representando por tanto los principales alérgenos. Sólo una minoría de perros normales mostraban una unión débil de IgE a estas proteínas. Los resultados indican que la mayoría de los perros atópicos con dermatitis por *Malassezia* tienen una respuesta de IgE mayor que los perros normales, sugiriendo que una respuesta inmune mediada por IgE puede ser clínicamente importante en la patogénesis de esta enfermedad.

Zusammenfassung Wir haben in der Vergangenheit nachgewiesen, dass normale Hunde und Hunde mit atopischer Dermatitis gegen *Malassezia pachydermatis*-Antigene eine IgG Antwort entwickeln. Das Ziel dieser Studie war, die mittels Western Blot gemessene IgE Antwort gegen Proteine von *M. pachydermatis* bei 28 atopischen Hunden mit *Malassezia*-Dermatitis und 22 klinisch normalen Hunden zu vergleichen. Sechs verschiedenen Entdeckungssystemen wurden untersucht, um die Sensitivität zu bestimmen und nicht-spezifische Bindungen und Kreuzreaktionen zu eliminieren. Das Protokoll mit den besten Ergebnissen benutzte einen monoklonalen, gegen Hunde-IgE gerichteten Mausantikörper, einen mit alkalischer Phosphatase konjugierten Ziegenantikörper gegen Maus IgG, der dreimal durch die Säule mit Hunde IgG geschickt wurde, ein chemilumineszentes Substrat und ein digitales Bildsystem. Proteine mit einer Masse von 45, 52, 56 und 63 kDa wurden von mehr als der Hälfte der atopischen Hundeseren gebunden und repräsentierten daher wichtige Allergene. Eine Minderheit der normalen Hunde zeigte schwache IgE Bindung an diese Proteine. Die Resultate deuten daraufhin, dass die Mehrheit der atopischen Hunde mit *Malassezia*-Dermatitis im Vergleich zu normalen Hunden eine erhöhte IgE Immunantwort zeigen und dass eine IgE-medierte Immunantwort klinisch in der Pathogenese der Erkrankung wichtig sein könnte.

Failure of extracts from *Malassezia pachydermatis* to stimulate canine keratinocyte proliferation *in vitro*

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Abstract Epidermal hyperplasia is one of the major histopathological features seen in dogs with *Malassezia* dermatitis. The aim of this study was to investigate the effects of extracts and culture supernatants from *Malassezia pachydermatis* on the proliferation of canine keratinocytes. Keratinocyte cultures were established from normal dog skin, and cell monolayers were co-cultured with *Malassezia* extracts (prepared either with or without protease inhibitors) and supernatants derived from organisms grown in liquid culture. The proliferation of keratinocytes was measured using a colourimetric assay. Neither the culture supernatants nor the *Malassezia* extracts had significant effects on the proliferation rate of canine keratinocytes, regardless of whether protease inhibitors were present or not. The results indicate that the epidermal hyperplasia seen in *Malassezia* dermatitis is unlikely to be caused directly by secretion of products from the organism.

Keywords: canine, keratinocytes, *Malassezia*, proliferation assays.

INTRODUCTION

Malassezia dermatitis is a disease that is increasingly recognized in dogs and its clinical features have been well documented.^{1–3} Skin biopsy samples from affected dogs generally show superficial perivascular-to-interstitial dermatitis, with hyperkeratosis, irregular hyperplasia of the epidermis and follicular infundibula, epidermal spongiosis and lymphocyte exocytosis.^{4–8} Scott and Miller⁴ described a hyperplastic dermatosis associated with secondary *Malassezia pachydermatis* infection in West Highland White Terriers. More recently, the epidermal dysplasia in two West Highland White Terriers was reported to be reversible after treatment, including antifungal therapy.⁹

A preliminary report has demonstrated a significant increase in a cellular proliferation marker in cultured canine keratinocytes infected with *M. pachydermatis* compared to noninfected cells.¹⁰ However, the mechanisms by which *Malassezia* might induce keratinocyte proliferation are not understood. One hypothesis for the epidermal hyperplasia seen in *Malassezia* dermatitis is that a product within, or secreted from, the organism may have a direct stimulatory effect on keratinocyte proliferation.

The aim of this study, therefore, was to evaluate the direct effects of extracts and culture supernatants from *M. pachydermatis* on the proliferation rate of cultured canine keratinocytes.

MATERIALS AND METHODS

Extracts of Malassezia pachydermatis

A strain of *M. pachydermatis* originating from the ear canal of a dog with *Malassezia* otitis was cultured on Sabouraud Dextrose agar (Oxoid, Basingstoke, UK) containing 20 mg mL⁻¹ chloramphenicol (Intramycin; Parke-Davis Veterinary, Pontypool, UK) for 48 h at 37 °C. *Malassezia* colonies were then carefully harvested and suspended in phosphate-buffered saline (PBS, pH 7.4). After three washing cycles with PBS, the cells were resuspended in extraction buffer (pH 7.4) comprising 125 mM NH₄HCO₃ (Sigma, Poole, UK) with or without protease inhibitors [20 mM ε-aminocaproic acid (Sigma); 5 mM ethylenediaminetetraacetic acid, (Sigma); and 1 mM phenylmethylsulphonyl fluoride, (BDH, Poole, UK)] for extraction of *Malassezia* proteins.^{11,12} The *Malassezia* colonies in the extraction buffer were then mixed vigorously with an equal volume of glass beads (0.4 mm, 40 mesh; BDH) on a vortex mixer for 10 min to mechanically disrupt the cell membranes. After extraction, the cell suspensions were stored at 4 °C overnight, centrifuged at 6000 g for 5 min and the supernatants were collected. The extracts were sterilized by passing through syringe filters with a 0.8, 0.45 and 0.2 µm pore-size serially. A 50-µL aliquot of the filtered extracts was inoculated on Sabouraud Dextrose agar (Oxoid) and the plate was incubated for 3 days at 37 °C to check its sterility. No growth of any organisms was observed after the 3-day incubation. The amount of protein obtained was measured with BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL, USA). The protein profiles of *Malassezia* extracts with or without protease inhibitors were demonstrated

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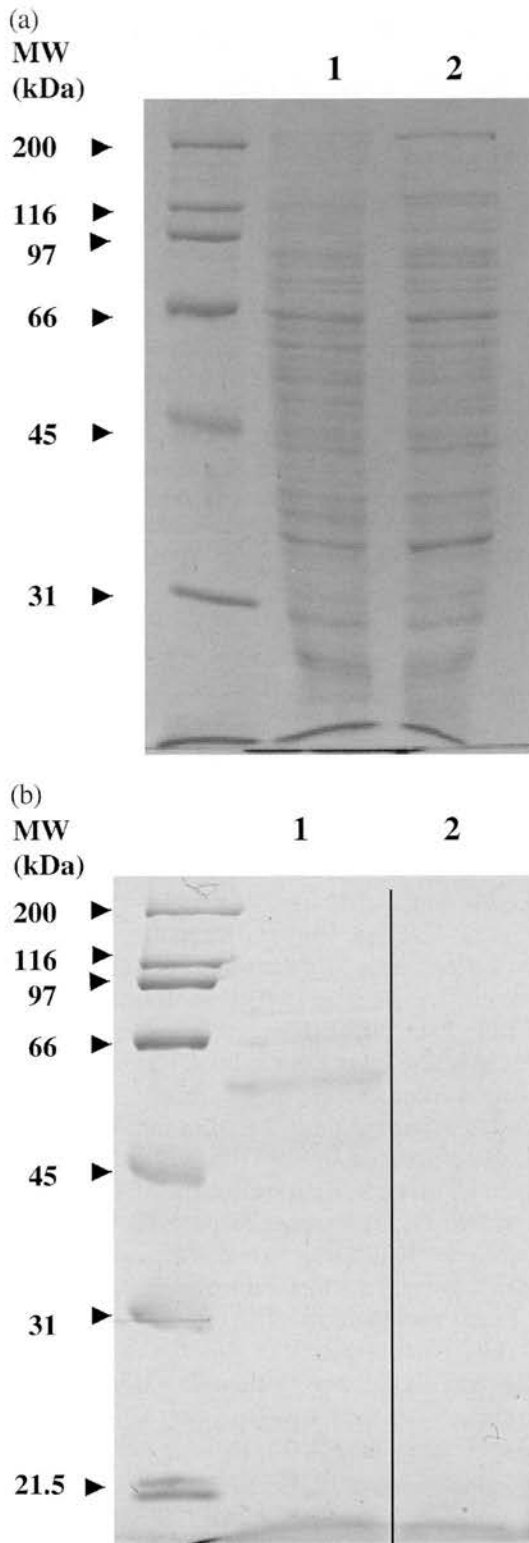


Figure 1. Coomassie Brilliant Blue stained SDS-PAGE gel. (a) Extracts of *Malassezia pachydermatis*. Lane 1, *Malassezia* extracts produced with protease inhibitors. Lane 2, *Malassezia* extracts produced without protease inhibitors. (b) Protein profile of culture supernatants from *M. pachydermatis*. Lane 1, culture supernatants from *Malassezia*; faint bands are visible. Lane 2, sterile Sabouraud liquid medium; no bands are visible.

using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with reducing sample buffer containing 10% β -Mercaptoethanol (Sigma) and are shown in Fig. 1(a).

Culture supernatants of Malassezia pachydermatis

Culture supernatants of *M. pachydermatis* were prepared by growing the organisms in Sabouraud liquid medium (Mast Laboratories Ltd, Merseyside, UK) for 3 days. The cell suspension was then centrifuged and the supernatant collected. It was concentrated using Centriplus concentrators (Millipore Corporation, Bedford, MA, USA) to a final volume of 2 mL and passed through syringe filters with a 0.2- μ m pore size. Sterile medium was also incubated and concentrated in parallel to act as a control. The protein profile of the culture supernatants was checked using an SDS-PAGE gel as described above (Fig. 1b).

Keratinocyte culture

Healthy skin was taken immediately post-mortem from dogs presented for euthanasia from a dog rescue centre. The flank of dogs was shaved and scrubbed with Hibiscrub, painted with Povidone-Iodine and wiped with 70% ethanol. Full-thickness skin was removed and dipped in Povidone-Iodine/PBS solution (1:5) for 10 s and then washed with PBS containing Penicillin (100 units mL⁻¹), Streptomycin (100 μ g mL⁻¹), and Fungizone (2.5 μ g mL⁻¹) (PSF; Life Technologies, Paisley, UK). The skin was then immersed in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) containing PSF.

Canine keratinocyte culture was established using a modification of a protocol published previously.¹³ Briefly, the subcutaneous tissue and fat were removed by trimming and the skin was cut into small pieces. The pieces were then incubated overnight at 4 °C in William's Medium E (WME; Life Technologies) containing 10 mg mL⁻¹ dispase (Life Technologies) and PSF. The epidermis was carefully stripped off the dermis and placed in a solution of trypsin/EDTA (0.1%/0.04%) (Life Technologies) at 37 °C for 10 min. The enzymatic effect of trypsin was abolished by adding an excess of WME supplemented with 10% foetal calf serum (FCS; Sigma), 0.1 nm cholera toxin (CT; Sigma), 10 ng mL⁻¹ murine epidermal growth factor (mEGF; Life Technologies), and PSF (complete medium). The cell suspension was filtered with a cell strainer to remove hair and cell debris. It was then centrifuged at 700 g for 10 min and the cells were resuspended in complete medium. For primary cultures, tissue culture flasks coated with fibronectin (kindly provided by Dr R. C. McKenzie, University of Edinburgh, UK) at a concentration of 1 μ g cm⁻² were used. Cells of passages 2–5 were utilized in the following experiments.

The morphological and cytoskeletal characteristics of the keratinocytes were verified using an immunostaining technique with monoclonal antibroad spectrum keratin and monoclonal antivimentin antibodies (EURO/DPC Ltd, Gwynedd, UK). Briefly, the cells were cultured in chamber slides to form a monolayer and then fixed with 4% paraformaldehyde in PBS for 20 min. After washing with PBS three times, cells were treated with 1% H₂O₂ in methanol for 20 min to block

endogenous peroxidase activity followed by another wash. They were then incubated with 1% SDS in PBS for 5 min as an antigen retrieval step to enhance staining. The slides were rinsed with PBS and blocked with horse serum for 1 h (Vector Elite ABC mouse IgG kit; Vector Laboratories Ltd, Peterborough, UK). An antikeratin or antivimentin antibody was added as the primary antibody and allowed to react for a further 1 h. The reagents in the IgG kit, including a biotinylated horse antimouse IgG and avidin/biotinylated peroxidase complex, were then used according to the manufacturer's instructions and each incubation step lasted for 30 min. The slides were developed using DAB (3, 3'-diaminobenzidine; Vector Laboratories Ltd) and counterstained with Mayer's haematoxylin (Sigma). Figure 2 shows the keratinocytes stained with antikeratin and antivimentin antibodies. Keratinocytes reacted to antikeratin antibody and exhibited brown staining (Fig. 2a). In contrast, they stained negatively with antivimentin antibody (Fig. 2b).

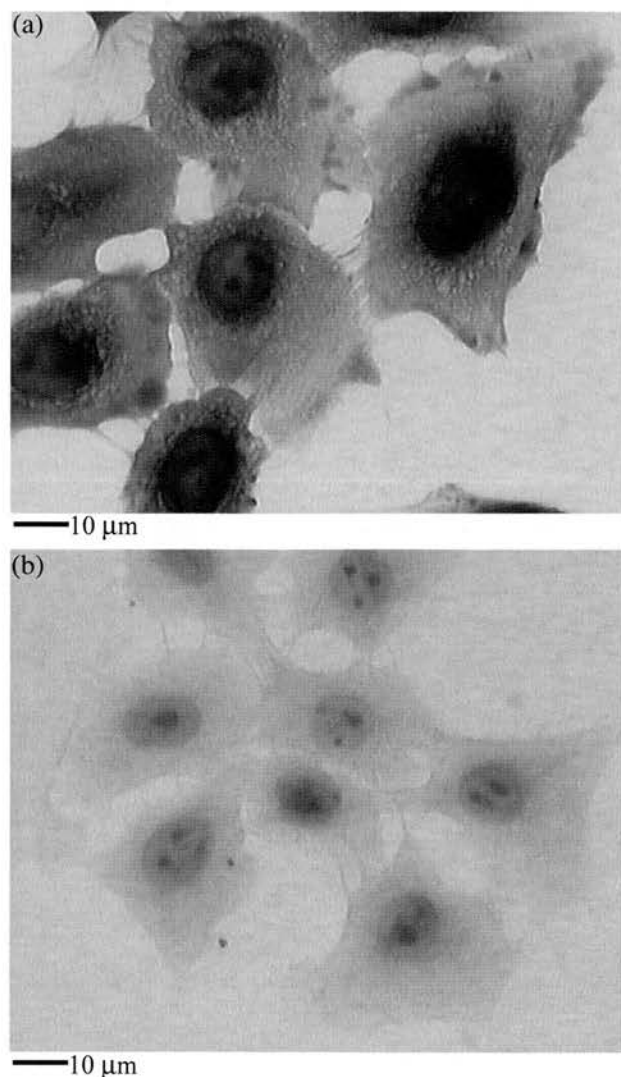


Figure 2. Cytoskeleton staining in canine keratinocytes ($\times 500$). (a) Anti-keratin antibody stained keratinocytes showing dark brown staining. (b) Keratinocytes appear unstained with antivimentin antibody. Scale bars = 10 µm.

Validation of a cell proliferation assay to detect keratinocyte proliferation

Cells harvested from tissue culture flasks were resuspended in WME and counted in a haemocytometer. Various combinations of supplements, including FCS, cholera toxin, and murine epidermal growth factor, were added to the concentrations of 10%, 0.1 nM, and 10 ng mL⁻¹, respectively. The cells were then plated into 96-well plates in quadruplicate (100 µL/well, 2×10^4 cells cm⁻²), and the plates were cultured at 37 °C with 5% CO₂ in air for up to 7 days. Cells from the same passage were also plated in flasks to be used for generating a standard curve in the cell proliferation assay.

A colourimetric assay reagent (Celltiter 96 AQueous One Solution Cell Proliferation Assay; Promega, Southampton, UK) was used to determine the number of viable keratinocytes. For the standard curve, cells grown in the flasks were trypsinized, washed and counted in a haemocytometer. A volume of 100 µL of cell suspension (containing twofold dilutions from 2×10^5 to 3125 cells per well) was added in triplicate to empty wells on the day of the assay. Wells with 100 µL of medium only were included as a background control. Twenty microliters of Celltiter 96 AQueous One Solution reagent was then added to all wells and the plates were incubated for a further 3 h. The absorbance of the reaction was evaluated using an ELISA reader (Dynatech MR5000, Dynatech Laboratories Ltd, Billingshurst, UK) at a wavelength of 490 nm and a reference wavelength of 630 nm. Optimum plating cell densities in tissue culture plates and assay conditions were established in preliminary experiments (data not shown).

A linear correlation was found between the absorbance and cell number per well (between 2×10^5 and 3125 cells per well) when both parameters were transformed to logarithms (Fig. 3). Therefore, this assay was judged to be a valid method for measuring canine keratinocyte proliferation *in vitro*.

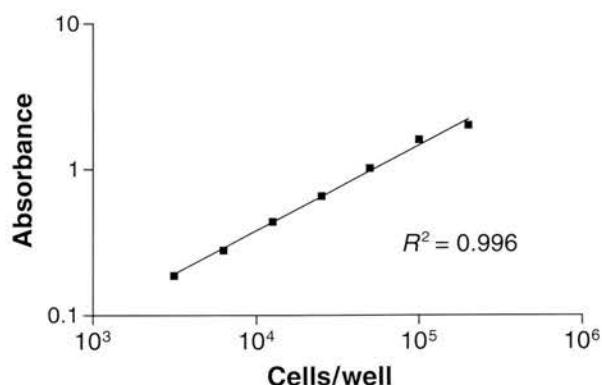


Figure 3. Correlation between cell numbers and absorption in the Celltiter 96 colourimetric assay. The absorbance is proportional to cell number between 2×10^5 and 3125 when both *x*- and *y*-axes are transferred to logarithmical scales. Data shown are the mean of triplicates, but the error bars are so small they cannot be displayed on the figure. *x*-axis, cell number per well; *y*-axis, absorbance at 490 nm with a reference wavelength of 630 nm.

Culture of keratinocytes with extracts and culture supernatants from *M. pachydermatis*

Keratinocytes were seeded into 96-well plates at 2×10^4 cells cm^{-2} in complete medium, as well as into flasks to be used for generating a standard curve in the cell proliferation assay. After 24 h, the medium was changed in order to remove nonadherent cells. Fresh medium and *Malassezia* extracts made with and without protease inhibitors were added into wells at a final concentration of 5×10^{-1} – 5×10^{-5} mg mL^{-1} . Wells containing medium alone and PBS served as controls. Concentrated *Malassezia* culture supernatants and sterile Sabouraud liquid medium were diluted 10-fold with PBS and used at final dilutions of 1/10 to 1/10 000. All wells were plated in at least quadruplicate for each experiment, and the plates were incubated for 3 days. Wells were washed and filled with 100 μL of fresh medium on the day of the assay and the number of viable cells in each well was measured using the colourimetric cell proliferation assay described above. Results of repeated experiments were combined to give more replicates for each group in subsequent analyses.

Data analysis

Results were expressed as the number of cells per well. The number of keratinocytes cultured with or without *Malassezia* extracts was compared using one-way ANOVA (Prism; Graphpad Inc., San Diego, USA). *T*-tests (Prism) were used to compare the cell numbers of keratinocytes incubated with culture supernatants of *Malassezia* and sterile medium for each dilution ratio. Significance was set at $P < 0.05$.

RESULTS

Effects of growth factors on keratinocyte proliferation

To verify that the culture conditions for keratinocytes were optimal, the cell proliferation assay was used to investigate the effects of growth factors on cell proliferation. Cells were cultured in WME alone; with WME and FCS; with WME, FCS and CT; with WME, FCS and mEGF; and with WME, FCS, CT, and mEGF (Fig. 4). Fewer cells were detected in wells containing only unsupplemented WME in comparison to the

other four groups. Cells grown in the complete medium (containing FCS, CT, and mEGF) had the highest proliferation rate, with more cells detected than the other groups in all three assays performed during the 7-day incubation. The findings are in accordance with those of an earlier study using manual counting with a haemocytometer.¹³

Effects of *Malassezia* extracts on keratinocyte proliferation

In order to examine the role of proteases in keratinocyte proliferation as well as other proteins in the *Malassezia* extracts, the extracts were made using buffer both with and without protease inhibitors. There was no significant difference in cell numbers between any group of keratinocytes cultured with 5×10^{-1} – 5×10^{-5} mg mL^{-1} of extracts, either with or without protease inhibitors, or PBS, which was the negative control ($P > 0.05$) (Fig. 5a,b).

Effects of culture supernatants from *M. pachydermatis* on keratinocyte proliferation

The results of culturing keratinocytes with culture supernatants from *Malassezia* are shown in Fig. 6. No significant difference was found between each group of cells incubated with culture supernatants from *M. pachydermatis* at the four dilutions compared to the individual negative controls ($P > 0.05$).

DISCUSSION

The purpose of this study was to determine if extracts and culture supernatants from *Malassezia pachydermatis* could induce proliferation of canine keratinocytes. This was suggested by a previous study using live organisms.¹⁰ Our findings, however, showed that *Malassezia* extracts did not affect keratinocyte proliferation with extracts produced either with or without protease inhibitors. These results suggest that products from *M. pachydermatis* do not contribute directly to the epidermal hyperplasia seen in *Malassezia* dermatitis.

Similarly, the proliferation of keratinocytes was not affected by coculturing keratinocytes with culture supernatants from *Malassezia pachydermatis*. We

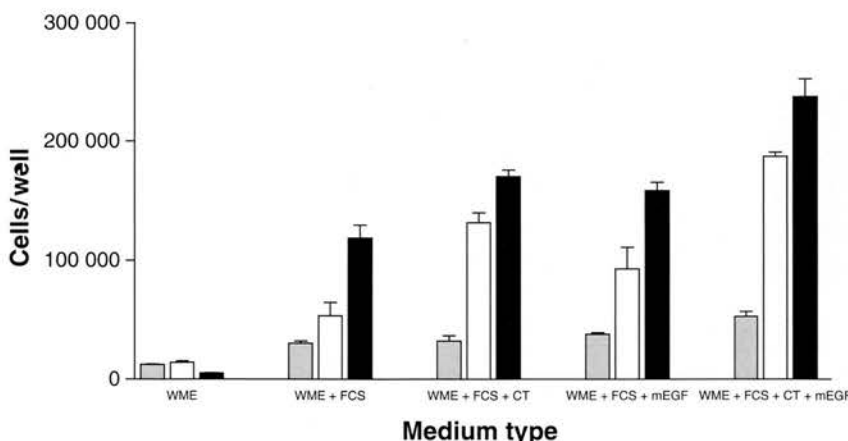


Figure 4. Comparison of the proliferation rate of keratinocytes cultured in William's Medium E with and without supplementation. Each bar represents the mean and standard error of the mean of quadruplicates. Shaded bars, day 3; open bars, day 5; black bars, day 7. WME, William's medium E; FCS, foetal calf serum; CT, cholera toxin; mEGF, murine epidermal growth factor.

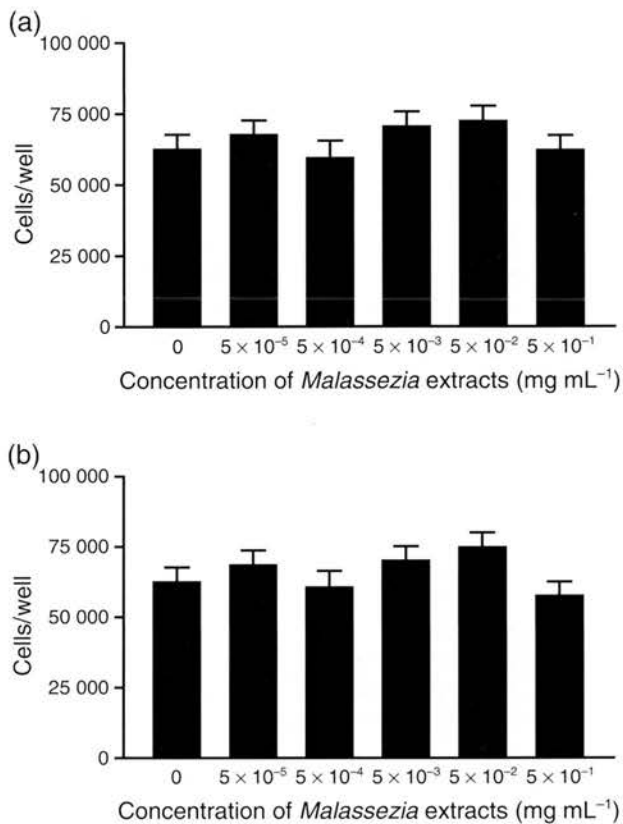


Figure 5. Dose-response of canine keratinocytes to *Malassezia* extracts. (a) Extracts produced using buffer containing protease inhibitors. (b) Extracts without protease inhibitors. Data shown are the combination of five separate experiments and the differences between groups were analysed using one-way ANOVA. No significant difference was found between groups.

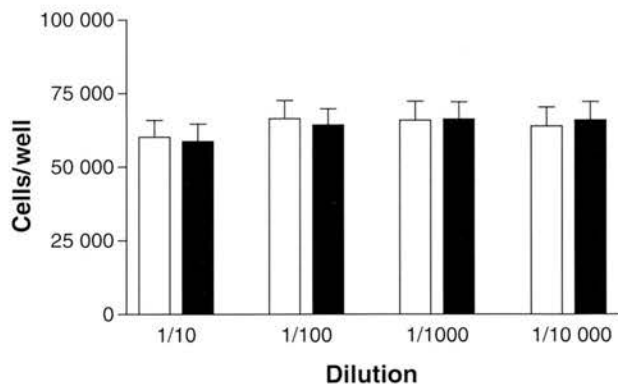


Figure 6. Dose-response of keratinocytes to culture supernatants from *Malassezia pachydermatis*. Data shown are the combination of four experiments and the differences between cells cultured with supernatants and sterile medium were analysed using *t*-tests. There was no significant difference between the two groups at any dilution. Open bars, sterile medium; black bars, culture supernatants from *Malassezia*.

showed that proteins with molecular weights between 66 and 45 kDa (Fig. 1b) were generated in the liquid medium or released from *Malassezia* during culture. It is unclear what these products are without further purification and analysis. However, protease activity has been demonstrated in yeast-free supernatants when *M. pachydermatis* was cultured in liquid medium.¹⁴ It is

likely therefore that the culture supernatants used in this study also contained proteases. Enzyme secretion is one of the virulence factors possessed by many micro-organisms. Protease is an important virulence factor of candidiasis in humans,^{15,16} and a keratinolytic protease of *Candida albicans* has been shown to digest human stratum corneum *in vitro*.¹⁷ Enzymes produced by *Malassezia* are generally considered to be potential pathogenic factors. Coutinho and Paula¹⁸ demonstrated that all the strains of *Malassezia pachydermatis* they studied isolated from dogs with otitis and dermatitis showed protease activity. Protease released by *Malassezia* was proposed as the mediator of itch at free nerve endings in the skin and a contributor to the prominent pruritus seen in affected dogs.² However, little work has been undertaken looking at the role of proteases in the pathogenesis of histopathological changes associated with *Malassezia* dermatitis. We found no difference in the growth rate of keratinocytes cultured with *Malassezia* extracts produced either with or without protease inhibitors and keratinocytes cultured with supernatants from *M. pachydermatis* compared to sterile medium. These results indicate that proteases or other proteins secreted by the organism do not cause changes in keratinocyte proliferation *in vitro*. If *Malassezia* can induce keratinocyte proliferation directly, it is likely that cell-to-cell interaction is required between the whole organism and the keratinocyte. This hypothesis requires further study.

This study was performed using healthy dog skin. It is important to consider the possibility that there may be a difference in proliferation rate in response to *M. pachydermatis* between normal and *Malassezia*-infected skin. Higher thymidine incorporation has been demonstrated in basal cells of *Candida*-infected skin compared to normal skin of guinea pigs.¹⁹ Whether this is the case in dogs with *Malassezia* dermatitis remains to be investigated. It is also possible that skin from dogs with atopic dermatitis might exhibit different proliferative responses to those of normal dogs, although this awaits further study.

In conclusion, the present study has shown that *Malassezia* extracts, either with or without protease inhibitors, do not cause keratinocyte proliferation *in vitro*. Likewise, culture supernatants from *Malassezia* had no effect on the growth rate of keratinocytes. It seems likely therefore that the excessive epidermal proliferation associated with *Malassezia* dermatitis is induced by other mechanisms.

ACKNOWLEDGEMENTS

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Résumé L'hyperplasie épidermique est un des aspects majeurs observé à l'examen histopathologique de chiens atteints de dermatite à *Malassezia*. Le but de cette étude était d'évaluer les effets d'extraits et de surnageants de cultures de *Malassezia pachydermatis* sur la prolifération kératinocytaire. Des cultures de kératinocytes ont été réalisées à partir de la peau de chiens sains, et des monocouches cellulaires ont été incubées avec des extraits de *Malassezia* (préparés avec ou sans inhibiteurs de protéases) et le surnageant obtenu à partir d'organismes cultivés en phase liquide. La prolifération kératinocytaire a été mesurée par une technique de colorimétrie. Ni les surnageants de culture, ni les extraits de *Malassezia* n'ont présenté un effet sur le taux de prolifération des kératinocytes, que ce soit en présence ou en absence d'inhibiteurs de protéases. Ces résultats indiquent que l'hyperplasie épidermique observée dans les dermatites à *Malassezia* n'est probablement pas liée à des produits de sécrétion des levures.

Resumen Epidermal hyperplasia es una de las características histopatológicas principales vistas en perros con el dermatitis de *Malassezia*. La puntería de este estudio era investigar los efectos extractos y los supernatants de la cultura de los *Pachydermatis de Malassezia* en la proliferación de keratinocytes caninos. Las culturas de Keratinocyte con fueron establecidas de piel normal del perro, y los monolayers de célula co-fueron cultivados los extractos de *Malassezia* (preparados con o sin los inhibidores del protease) y los supernatants derivados de los organismos crecidos en cultura líquida. La proliferación de keratinocytes fue medida usando un análisis colourimetric. Ni los supernatants de la cultura ni los extractos de *Malassezia* tenían efectos significativos en el índice de la proliferación de keratinocytes caninos, sin importar si los inhibidores del protease estaban presentes o no. Los resultados indican que la hiperplasia epidérmica vista en el dermatitis de *Malassezia* es poco probable ser causado directamente por la secreción de productos del organismo.

Zusammenfassung Epidermale Hyperplasia ist eine der wesentlichen histopathologischen Veränderungen der *Malassezia*-Dermatitis beim Hund. Das Ziel dieser Studie war es, die Wirkung von Extrakten und vom Kulturüberstand auf die Proliferation von Hundekeratinozyten zu untersuchen. Keratinozyten von normaler Hundehaut wurden kultiviert und Zellmonoschichten wurden mit *Malassezia*-Extrakten (entweder mit oder

ohne Proteasehemmer zubereitet) und mit Kulturüberstand von in flüssiger Kultur gewachsenen Organismen gezüchtet. Die Keratinozytenproliferation wurde mittels eines kolorimetrischen Assays gemessen. Weder der Kulturüberstand noch die *Malassezia*-Extrakte (unabhängig von der Anwesenheit eines Proteinasehemmers) hatten signifikanten Einfluss auf die Proliferationsrate der Keratinozyten. Diese Ergebnisse deuten darauf hin, dass die bei *Malassezia*-Dermatitis gesehene epidermale Hyperplasie wahrscheinlich nicht direkt durch Sekretionsprodukte des Organismus verursacht wird.