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THE SKIN IMMUNE RESPONSE TO *MALASSEZIA FURFUR*

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Submitted for the Doctorate of Philosophy

The University of Glasgow

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ABSTRACT

The paradoxical nature of *M. furfur* colonisation versus infection in dermatological disease is subject to much research. The aim of this experimental work was to measure the innate immune response of the skin to *M. furfur*, via the use of skin models. Living skin equivalents (LSE), excised breast reduction tissue (BRT) and keratinocyte (KC) monolayers were all utilised in an attempt to elucidate the possible immune evasive and stimulatory capabilities of *M. furfur*.

The constitutive production of human β defensin 2 (HBD-2), an inducible antimicrobial peptide, was measured KC monolayers, LSEs and BRT. In addition the skin model's response to fungal challenge was elucidated. Wide variation in the basal expression of HBD-2 was detected in all skin model donors. *M. furfur* cell wall and killed whole *M. furfur* initiated a slight depression in HBD-2 expression by KC monolayers, however these results were not statistically significant in all donors and merely indicated a trend. Likewise KC monolayers, BRT and LSEs reacted to viable *M. furfur* with slight inhibition of HBD-2 production at 24hr with subsequent stimulation of expression. However donor variation in this pattern was detected and these results were not continuously significant. Due to the non-continuous nature of these measurements these results were inconclusive

As *M. furfur* infection of the skin is associated with alteration of the normal pigmentation in patients, melanin synthesis by B16 mouse melanoma cells and BRT was assessed in response to *M. furfur*. Viable *M. furfur* and *C. albicans* stimulated an increase in melanin synthesis in B16 mouse melanoma cells. The ability of viable *M. furfur* cells to stimulate melanin synthesis appeared to be localised within the cytoplasm of the organism. However, this 'viable cell stimulation' did not appear to be restricted to *M. furfur*, as *C. albicans* also stimulated melanin synthesis. On BRT there was little difference in the melanin and tyrosinase production of BRT in reponse to *M. furfur* and *C. albicans* growth. The capacity of *M. furfur* and *C. albicans* cell wall to alter the cytokine profile of KCs was also measured and KC monolayers exhibited a time-dependent increase in IL-1α, IL-8 and ET-1 expression in response to *M. furfur* and *C. albicans* cell wall. *C. albicans* cell wall initiated a significantly greater increase in the expression of these cytokines by the KCs. However little correlation between the mRNA production and peptide production was measured

using RT-PCR.

Growth of *M. furfur* and *C. albicans* on the skin models was assessed using scanning electron microscopy (SEM) and histological observation of the colonized tissue. Growth was also compared by means of viable cell counts. The effect of growth on the proliferation of the epidermis was measured by counting the number of proliferating cells in the basal layer of the epidermis of each tissue. Growth of *M. furfur* and *C. albicans* was detected on LSE and BRT and hyphal transformation of both organisms was observed on BRT and LSE, although hyphal transformation of *C. albicans* was found more commonly on the LSE. Indeed, overall growth of *C. albicans* was more widespread and rapid on LSE than it was on BRT. By contrast *M. furfur* appeared to undergo hyphal transformation more frequently on BRT, and this feature was donor-dependent. The viability of *M. furfur* varied when tested on BRT from different donors or on different batches of LSE. The proliferative index of the tissues indicated that growth of both *M. furfur* and *C. albicans* initiated an increase in the proliferation of the BRT and LSE epidermis. Overall, these studies show that growth of *M. furfur* and *C. albicans* differs in the various skin models and this effect was dependent on the different qualities of the donor tissue and donor KCs. The growth of *M. furfur*, while slower than that of *C. albicans*, does stimulate a larger increase in the proliferation of the BRT epidermis.

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I would like to extend thanks to all my friends, both within the Department and externally for their support, emotional and pratical, during my PhD. In particular I would like to thank the Friday night contingent, without whom I would have never left the lab especially Pauline, Tracey, Filipa and Mike. Most importantly I would like to thank my family. My mum and dad have supproted me throughout my research, in so many ways, and I will be forever indebited for their contribution. I would also like to thank my brother and sister as their endless encouragement is directly responsible for the completion of this thesis.

DEDICATION

To Tony, Sean, Brendan and the girls (to come).

ABBREVIATIONS

AD	Atopic dermatitis
AIDS	Acquired immunodeficiency syndrome
AMU	Atomic mass unit
αMSH	Alpha melanocyte stimulating hormone
BAL	Bronchialveolar lavage fluid
BRT	Breast reduction tissue
bFGF	Basic fibroblast growth factor
CFU	Colony forming units
CXC	Chemokine
DEPC	Diethylpyrocarbonate
DHICA	5,6-dihydroxyindole
DOPA	3,4-dihydroxyphenylalanine
EDTA	diaminoethanetetra-acetic acid disodium salt
ET-1	Endothelin 1
FB	Fibroblast
FCS	Foetal calf serum
FISH	Fluorescent *in situ* hybridisation
GAPDH	D-glyceraldehyde-3-phosphate dehydrogenase
GPC	Glucose peptone chloramphenicol
hCAP-18	Human cationic peptide 18
HBD-2	Human beta defensin 2
H&E	Haematoxylin and eosin
HIV	Human immunodeficiency virus
HLA	Human lymphocyte antigen
HPLC	High performance liquid chromatography
HNP	Human neutrophil peptide
IL-1α	Interleukin 1 alpha
IL-8	Interleukin 8
ISD	Infantile seborrheic dermatitis
KC	Keratinocyte

LC	Langerhans cell
LPS	Lipopolysaccharide
LAP	Lingual antimicrobial peptide
LSE	Living skin equivalent
MMP	Matrix metalloproteinase
MC	Melanocyte
MEM	Minimal essential medium
Mwt	Molecular weight
NFκB	Nuclear factor kappa beta
PPRR	Pathogen pattern recognition receptor
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
PV	Pityriasis versicolor
RLU	Relative light units
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Seborrheic dermatitis
SLPI	Secretory leukoproteinase
SEM	Scanning electron microscopy
SIS	Skin immune system
TAP	Tracheal antimicrobial peptide
TBE	TrisborateEDTA
TBS	Tris-buffered saline
TLR	Toll-like receptor
TRP	Tyrosinase related protein
UV	Ultra violet

1 INTRODUCTION

1.1 MALASSEZIA FURFUR

1.1.1 HISTORY

The role of fungi in dermatological disease has been controversial with regard to the genus *Malassezia.* Yeast cells were first reported by Eichstedt (1846) and Robin (1853) in association with skin scrapings from pityriasis versicolor (PV). Subsequently Rivolta (1883) described yeast involvement in psoriasis and Malassez (1874) was the first to associate the yeast with scalp scaling. Much controversy surrounds the correct nomenclature for both genus and species of *Malassezia/Pityrosporum;* the latter term is still used, most predominantly by J. Faergemann (Faergemann, 1993). *Malassezia* has, however, been more widely accepted by the scientific community and in particular by Gordon who had initially adopted the name *P. orbiculare* to describe the organism he isolated from PV (Gordon, 1979). Until recently, controversy surrounded the distinction between what were considered to be two different species: *P. orbiculare* and *P. ovale.* By karyotyping it has been shown that these organisms represent two different morphological stages of the same organism (Gueho and Meyer, 1989). Hence the round *P. orbiculare,* oval *P. ovale* and mycelial *M. furfur* are in fact the same organism which can be grouped under the common nomenclature of *Malassezia furfur* (Table 1). Seven organisms are currently identified within the *Malassezia* genus including *M. furfur, M. pachydermatis, M. sympodialis, M. globosa, M. obtuse, M. restricta* and *M. slooffiae* (Boekhout et al., 1998). The majority of these organisms are implicated in the pathogenesis of veterinary infections, particularly *M. pachydermatis* and *M. sympodialis* in otitis externa. Due to the previous confusion surrounding nomenclature, the naming of *Malassezia* organisms in disease has been widely misinterpreted. It is, however, widely accepted that *M. furfur* is the primary human pathogen, implicated in a range of human skin and systemic infections.

Table 1: Classification of *Malassezia* species.

ORGANISM	FEATURES	PATHOGENICITY
M. furfur	Lipophilic, dimorphic.	Mycelial stage of organism associated with PV Seborrheic dermatitis (SD), dandruff, and an allergen for atopic dermatitis etc.
M. pachydermatis	Non-obligatory lipophile. Small ovoid yeast cells.	Common veterinary pathogen, found also on human skin.
M. sympodialis	Lipophilic, dimorphic, oval/globose yeast cells.	Often implicated in systemic infections following lipid emulsion therapy.
M. globosa	Large, cylindrical, dimorphic yeast cells.	Commonly associated with otitis externa in cattle.
M. obtuse	Large, cylindrical, dimorphic yeast cells.	Lipid-dependent organism recently associated with canine otitis.
M. restricta	Spherical/oval yeast cells with no evidence of budding.	Lipophilic organism identified as skin commensal in humans.
M. slooffiae	Short, cylindrical cells.	Also associated as a veterinary and human pathogen with otitis infection.

1.1.2 STRUCTURE/ GROWTH

M. furfur displays characteristics common to other yeast species. The organism can be observed in culture in both its oval and cylindrical forms, ranging in size from 2.5 to 5.0μm and 1.5 to 3.0 to 2.5 to 8.0μm respectively. Hyphal transformation can be induced *in vitro* by the addition of exogenous sterols, cholesterol and cholesterol stearate (Porro et al., 1977). Standard culture of the organism requires the addition of long chain, C_{12}-C_{24}, fatty acids as the organism cannot synthesise the lipids *de novo*, and they are essential for its membrane integrity (Porro et al., 1976). Optimum growth is achieved in the temperature range of 22-37^{0}C. The cell wall has been studied by electron microscopy revealing a glycan-rich structure with extensive invaginations on the periplasmic side with indentations into the plasma membrane and a distinct lamellar layer (Mittag, 1995). It has been hypothesized that one of the major allergens of *M. furfur*, the Malf-1 protein, is attached to the cell wall (Schmidt et al., 1997), possibly at the periplasmic side, and is revealed during

reproduction. As *M. furfur* is an obligatory lipophile it comes as no surprise that it produces both a soluble and insoluble lipase and lipoperoxidase that is essential for growth (De Luca et al., 1996).

1.1.3 ANTIFUNGAL SENSITIVITY

The *in vitro* antifungal sensitivity of *M. furfur* is broad ranging, as measured by various growth inhibition assays. Azoles-agents which interfere with membrane ergosterol biosynthesis by interference with the cytochrome P-450 enzyme, lanosterol-14-α demethylase-have been shown to be effective in inhibiting growth (Strippoli et al., 1997). *In vitro* activity has also been shown with climbazole (Schmidt, 1997), zinc pyrithione, selenium disulphide (Van Cutsem et al., 1990), piroctone-olamine (Pierard-Franchimont et al., 2000), itraconazole (Rhie et al., 2000), fluconazole and terbinafine (Gupta et al., 2000). Antifungal activity has also recently been attributed to the 1,8-cineole-1-terpinen-4-ol component of tea tree oil (Hammer et al., 1997). It is also interesting to note the *in vitro* sensitivity of *M. furfur* to androgenic steroids, testosterone and dehydropoandrosterone; by contrast these compounds have no activity on *C. albicans* (Brasch, 1993). This finding is not consistent and conflicts with the effect *in vivo* as there is an accumulation of these androgenic steroids in the pilosebaceous unit, which is thought to be the site of *M. furfur* colonisation in SD. This study points out the disparity between *in vitro* and *in vivo* antifungal sensitivity and highlights the danger of extrapolating *in vitro* data for *in vivo* usage.

1.1.4 HABITAT

M. furfur can be isolated from the scalp and skin of the majority (74-100%) of the normal healthy population (Roberts, 1969). There is a greater frequency of colonisation of the sebaceous areas of the back and chest as has been shown by comparative quantification of organism numbers isolated from skin scrapings at both sites (Abraham et al., 1987; Faergemann, 1984). This is also supported by the increase in infection in adolescents when lipid production by the sebaceous glands is at its peak (Powell et al., 1984). In infants it has been shown that colonisation of the arms, back and chest affects approximately 32% of the neonatal population (Broberg, 1995; Broberg and Faergemann, 1989). The source of infant colonisation is thought to be either health care workers (Chang et al., 1998) or parents (Ahtonen et al., 1990), and length of hospital stay directly correlates to increased frequency

of colonisation (Bell et al., 1988).

1.1.5 PATHOGENICITY

As *M. furfur* can be isolated from various body sites of a large percentage of the population, it has been difficult to establish the role of this commensal in dermatological disease. Evidence for the organism's role in various cutaneous infections comes primarily from the correlation between disease resolution and response to antifungal treatment.

1.1.5.1 PITYRIASIS VERSICOLOR

The clinical presentation of this infection is characterised by chronic scaling of the upper trunk, chest, back and neck, often accompanied by hypo- and hyper pigmentation of the affected area (Faergemann, 1989). The lesions often coalesce to form large areas with abnormal pigmentation, with the colour of the lesion dependent upon exposure of the area to sunlight and the underlying pigmentation of the individual (Karaoui et al., 1981). The highest incidence of this infection occurs in areas of high relative humidity, which correlates to increased frequency of infection in tropical climates (Kim et al., 1999). Due to the predisposition of the organism for lipid, it is hardly surprising that the areas most affected are those with elevated lipid content, namely the sebaceous regions of the body, trunk and back. This corresponds to the elevated rate of infection in post-pubertal males who have high sebum secretion rates (Noble and Midgley, 1978). Clinical diagnosis is made by distribution and appearance of lesions, confirmed by fluorescence of infected areas under a Wood's lamp. Skin scrapings also yield culturable *M. furfur* and KOH digestion of samples should reveal the organism in both yeast and mycelial forms, commonly referred to as 'spaghetti and meatballs' (Faergemann et al., 1983b). In biopsies of infected areas the organism is confined to the epidermis and is generally observed in the stratum corneum with a mild mononuclear infiltrate in the upper epidermis often evident. Increased pigmentation in the basal layers and hyperkeratosis are also features (Janaki et al., 1997). It has been shown recently that the organism, although restricted to the stratum corneum, can elicit an increase in IgG and IgM-specific responses compared to unaffected (non-PV+ve) individuals (Silva et al., 1997). Treatment for PV includes synthetic detergent with added antifungal agents including ketoconazole (Borelli, 1980), zinc pyrithione and selenium sulphide at 2-5% (Chu, 1984). In chronic cases prophylaxis with fluconazole (Strippoli et al., 1997), itraconazole (Galimberti et al., 1987) and ketoconazole (Meisel,

1983) must be used to ensure non-recurrence.

Controversy surrounds the ability of the organism to alter pigmentation in PV. It has been hypothesised that inhibition of tyrosinase production (Nazzaro-Porro and Passi, 1978), disruption of melanosome distribution and size (Karaoui et al., 1981) and phospholipase production (Riciputo et al., 1996) may all be organism-derived factors that alter pigmentation. It has not been established which of these mechanisms, if any, predominate in the control of pigmentation changes seen in PV.

1.1.5.2 MALASSEZIA FOLLICULITIS.

Folliculitis, resulting from *M. furfur* infection, presents as an erythematous papulopustular rash commonly found on the back, shoulders, anterior chest and neck (Klotz et al., 1982). It is thought that the organism plugs the pilosebaceous unit, where budding cells are found in the infundibulum, resulting in follicle destruction and perifollicular inflammation. Puritis and crusting of the lesion leads to a chronic infection (Ford, 1984) and diagnosis is made on clinical presentation and histopathology of the infected follicles (Lim et al., 1988). The efficacy of antifungal agents, including selenium sulphide shampoos, ciclopirox and topical azoles (Parsad et al., 1998), all point towards fungal aetiology and can aid in the differential diagnosis between acne vulgaris and acneiform exanthema (Sandin et al., 1993). Again an association between host immune status and *M. furfur* infection has been indicated by the latest folliculitis study (Rhie et al., 2000), further supporting a role for the immune response in regulating the pathogenicity of this commensal yeast.

1.1.5.3 SEBORRHEIC DERMATITIS

SD presents as a yellow/ brown greasy scaling most often affecting the scalp, but also found on the face and upper trunk (Bergbrant, 1995). The link between *Malassezia* and SD has only recently been established in treatment studies showing the efficacy of antifungals in disease resolution (Heng et al., 1990; Skinner et al., 1985) corresponding to declining frequency of colonisation (Shuster, 1984). SD affects 1-3% of the general population, accounting for 7% of all dermatological out patients and presenting in 20-83% of patients with acquired immunodeficiency syndrome (AIDS) (Schechtman et al., 1995b). Although clinical diagnosis is often facilitated by the obvious characteristics of infection, differential diagnosis must also be made between psoriasis vulgaris, PV and histocytosis X in infants.

Dandruff is a mild non-inflammatory form of SD confined to the scalp (Hay and Graham-Brown, 1997; Priestley and Savin, 1976; Shuster, 1984) which is treated effectively using one of the many commercial applications (Pierard-Franchimont et al., 2000). Histologically the disease presents as hyperkeratosis with increasing frequency of nucleated corneocytes in the upper epidermis and a significant increase in scalp colonisation with *M. furfur* (McGinley et al., 1975). The increasing incidence of SD in AIDS patients (Aly and Berger, 1996; Ross et al., 1994) can be correlated with a declining T-cell count (Figure 1); hence, it is thought that the loss of an immunological response in AIDS patients may contribute to the initiation of SD.

Recent immunological studies have shown that decreased IgG reactivity to protein extracts of *M. furfur* correlates with increased severity of SD (Kieffer et al., 1990); however, other authors have shown increased IgG and IgA production (Ashbee et al., 1994). Unchecked proliferation of the organism in the skin could induce inflammation, triggering the hyperkeratosis and increased KC proliferation seen in SD. Production of IL-2 and IFN-γ is found to be decreased in SD sufferers while there is a marked increase in CD4+ expression on infiltrating T-cells, and increased NK-cell circulation and IL-10 production (Bergbrant et al., 1991b). It has still not been established with any clarity whether *M. furfur* can initiate a primary immune response in skin; however, recent studies have shown that it is able to stimulate Langerhans cell (LC) maturation and migration, indicating its ability to stimulate the adaptive immune response (Buentke et al. 2000). Conventional SD treatment is 2% ketoconazole and this results in marked improvement in the symptoms of SD (McGrath and Murphy, 1991).

1.1.5.4 Infantile Seborrheic Dermatitis

Infantile SD presents in a similar way to the adult form of SD (Broberg and Faergemann, 1989). Greasy scales on the scalp thicken and compress to form a cap, which overlies mildly erythematous skin-hence the generalised use of the term 'cradle cap' (Ruiz-Maldonado et al., 1989). Involvement may also occur over the body with erythema particularly affecting the flexural folds and nappy area. It has been shown that infants with infantile SD share a common disruption in their essential fatty acid metabolism and that correction of this abnormality with maturation results in disease resolution (Tollesson et al., 1997). The role therefore of *M furfur* in this disease may be associated with an altered

lipid profile, encouraging growth of the organism (Broberg and Faergemann, 1989).

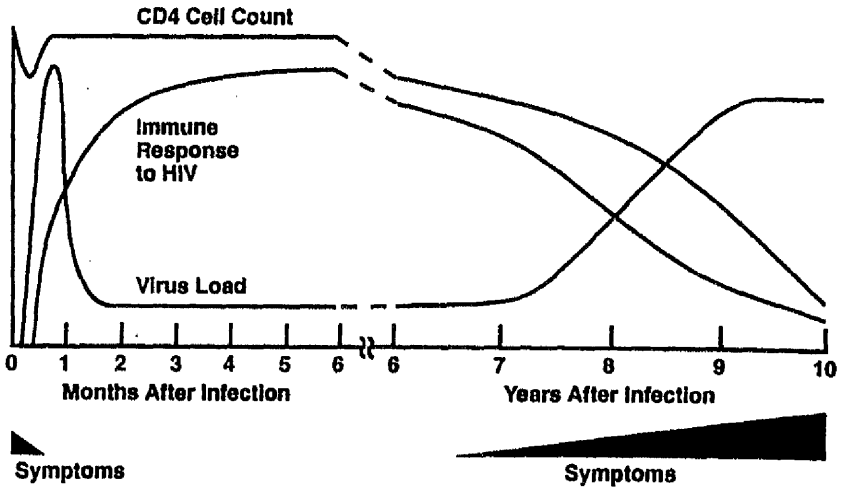


Fig. 1.a. Virology or natural history of HIV infection. (Courtesy of World Health Communications, New York, N.Y.)

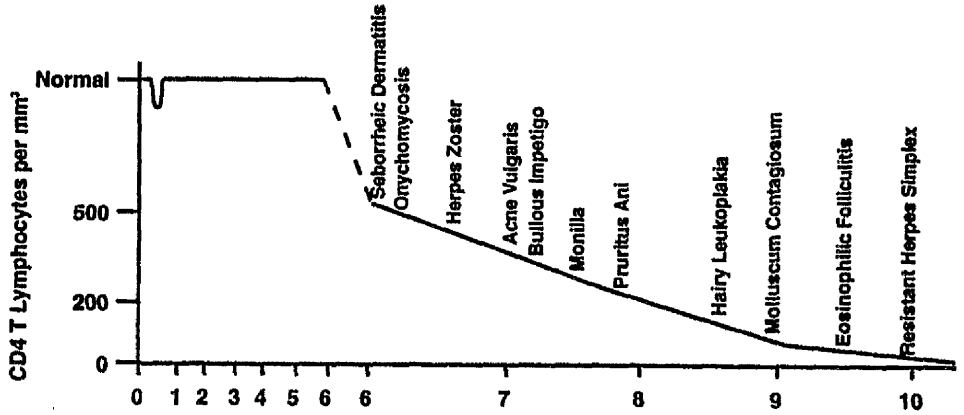


Fig. 1. b. Opportunistic skin infections during the course of HIV infection. (Courtesy of World Health Communications, New York, N.Y.)

Figure 1: Decreasing CD4 T-cell count (Fig. 1.a.) and onset of AIDS correlates to increased incidence in SD and other dermatological diseases (Fig. 1.b) (Faergemann, 1994).

1.1.5.5 Confluent and Reticulate Papillomatosis

This rare disorder, which generally affects young women, presents as grey-brown pigmented papules which coalesce, most commonly at the neck and upper trunk, to form hyper-pigmented patches (Faergemann et al., 1980; Kirby and Borrie, 1975). Although the

organism can be identified from lesions, its role in the initiation of infection is unclear (Kumar and Pandhi, 1984). It is thought that a genetically predisposed abnormal immune response towards *M. furfur* may be responsible (Broberg and Faergemann, 1988).

1.1.5.6 Atopic Dermatitis

The role of *M. furfur* as an allergen in individuals with AD has recently been assessed (Tengvall Linder et al., 2000). It has been established that the organism is an important allergen in individuals with AD localised to the face and neck (Waersted and Hjorth, 1985), as 78% of these patients show positive skin prick test reactivity to *M. furfur* extracts (Young et al., 1989) and positive skin prick test also correlates to efficacy of oral ketoconazole in AD resolution. In this *M. furfur*-sensitive population there is a greater frequency of IgE antibody specific to the organism, and serum shows elevated overall IL-4 and IL-10 levels (Jensen-Jarolim et al., 1992; Kroger et al., 1995). T-helper cells play an important role in the pathogenesis of AD, and allergen-specific T-cells isolated from individuals are often of the Th2 subclass. Treatment with antifungal agents does not, as in SD, correspond to disease resolution in general AD sufferers but does in individuals with AD localised to the head and neck (Broberg and Faergemann, 1995). Interestingly there appears to be a cross-reactive component of both *M. furfur* and *C. albicans* for atopics, who express a common and cross-reacting IgE response to these organisms (Zargari et al., 1994). How these commensals exacerbate AD is subject to much hypothesis, and it is possible that gastrointestinal sensitization to *C. albicans* growth in AD sufferers may result in a cutaneous reaction to *M. furfur* following exposure on the skin.

1.1.5.7 Psoriasis

Controversy also surrounds the putative role of *M. furfur* in the initiation of lesions in psoriasis. Animal models develop psoriatic-like lesions following application of *M. furfur* (Rosenberg et al., 1980) and, as *M. furfur* activates the alternative components of complement, this is a possible mechanism by which the organism could induce inflammation in psoriasis (Squiquera et al., 1994). It has been recently established that *Malassezia*-derived lipase stimulates release of arachidonic acid, a precursor for various pro-inflammatory agents from epithelial cell culture (Plotkin et al., 1988), and that a specific immunoglobulin response to this organism is detected in psoriatics but not in healthy individuals (Mathov et al., 1996). In some susceptible psoriatics *M. furfur*-derived

proteins may initiate an inflammatory immune response, resulting in plaque formation; this has been substantiated by the initiation of psoriatic plaques by *M. furfur*-derived components (Bunse and Mahrle, 1996; Lober et al., 1982). The inflammatory response initiated by the organism may accumulate, resulting in the hyperproliferative, neutrophil-rich plaque common to psoriasis. This susceptibility to *M. furfur* may be due to the genetic predisposition identified for psoriatics, where a mutation in the human lymphocyte antigen (HLA) gene shows a positive correlation to disease allowing inflammatory agents to exacerbate the already pre-existing condition (Baker et al., 1997).

1.1.6 SUMMARY

M. furfur is a lipophilic dimorphic yeast which is implicated, often inconclusively, in many dermatologic diseases. Much still has to be established regarding the relevance of this organism in these disorders, particularly the role it plays in the initiation of the immune response of the skin. An investigation into the immunomodulating activity of *Malassezia* on the skin may provide an insight into the organism's role in the pathophysiology of the infections mentioned above.

1.2 C. ALBICANS

C. albicans is a dimorphic yeast belonging to the genus *Candida*. *C. tropicalis* and *C. albicans* can be isolated from normal skin as part of the cutaneous microflora. The spectrum of human infection caused by *C. albicans* is vast; however, in relation to skin, this organism only becomes relevant when the host becomes severely immunocompromised. Chronic mucocutanous candidiasis is a group of symptoms characterized by chronic superficial *Candida* infection of the skin, nails and oropharynx. Increased incidence of *C. albicans* involvement in skin infection has been associated with a declining T-cell population, but as yet no distinct reversion of this commensal to pathogenicity has been identified during normal cutaneous colonisation. *C. albicans* is however implicated in onychomycosis, where the production of a keratinolytic proteinase has been identified as a pathogenic factor (Hattori et al., 1984) and, as *C. albicans* exhibits cross-reactivity with a *M. furfur*-specific antibody, there is a possible role for this yeast in aggravating cutaneous disease (Doekes et al., 1993). It is possible that the yeast stimulates the skin via the mannose receptor, beta- glucan receptor and complement receptor type 3,

which upon binding activates the alternative pathway of complement (Suzuki et al., 1998).

1.3 SKIN MODELS AND SKIN

1.3.1 HISTORY

Preliminary work on attempting to create 'skin models' originated from research on the interactions between the epidermal and dermal cells in co-culture as opposed to monolayers (Ponec and Kempenaar, 1995). Cell differentiation and proliferation mimic *in vivo* patterns of growth more representatively in three dimensional culture systems and these findings have revolutionised the field of tissue engineering (Stoppie et al., 1993). More recently the need for replacement tissues, not exclusively skin, has induced research in applied tissue engineering with specialisation in various tissue equivalents becoming more refined. The use, therefore, of LSEs for both toxicological and immunological studies has become more commonplace (LeClaire and de Silva, 1998).

1.3.2 STRUCTURE

The LSE is a three dimensional model with a viable stratified epidermis supported upon a fibroblast-contracted collagen matrix. Many different protocols for formulating a LSE have been devised, utilising hair follicle KCs, neonatal KCs, de-epidermised dermis and fibroblast-embedded collagen matrices (Lenoir and Bernard, 1990). The most commonly used protocol incorporates a fibroblast-embedded collagen matrix, seeded with neonatal KCs. This skin equivalent produces a differentiated stratified epidermis which expresses the appropriate keratin markers (Coulomb et al., 1998) with a distinct deposition of laminin and type IV collagen at the basement membrane (Sugihara et al., 1991). Although skin equivalents do represent normal skin in many respects they lack some cellular components, such as melanocytes (MCs) and LCs, which also contribute to tissue homeostasis (Asselineau et al., 1986). To utilize the LSE as an experimental model the investigator must be aware of the practical constraints of the model and compare the reactivity of the LSEs to the known activities of skin. Two areas in which the skin equivalent fails to be representative of real skin, and which are particularly relevant to *M. furfur* infections, are its lipid profile and immunological responses.

1.3.3 LIPID COMPOSITION

With reference to the stratum corneum, the lipid profile of the skin is high in ceramides, converted from glucosyl ceramides, long chain C_{22}-C_{30} free fatty acids, free sterols and cholesterol sulphates. The lipoprotein of corneocytes is cross-linked to the ceramides of the horny layer and recent studies have shown that the lipid content or ceramide profile of the LSE does not correspond to the *in vivo* level (Ponec et al., 2000). Hence the functioning of the LSE stratum corneum differs substantially from normal skin in that it is more absorbent, and has less of a barrier function than natural skin. It has recently been shown that the addition of vitamin C (50 μg/ml ascorbic acid in the medium) improves epidermal cell morphology and normalises the lipid content of the stratum corneum, and in particular the ceramide content (Ponec et al., 1997b). It is thought that by optimising the lipid profile of the skin equivalent, the balance between differentiation and proliferation exerted by ceramides and glucosyl ceramides respectively, will result in the normalisation of the desquamation rate of the LSEs stratum corneum (Vicanova et al., 1996). Other methods for normalising the lipid content of reconstructed epidermis involve supplementing the medium with essential fatty acids or reducing the relative humidity and temperature (Ponec et al., 1997a).

1.3.4 IMMUNOLOGICAL ACTIVITY

Although the basic structural, morphological and biochemical interactions of the skin model are equivalent to the situation found in normal skin, the lack of systemic humoral and cellular immunological responses makes the model limited in its immunological relevance. Although certain cytokine profiles have been measured (de Brugerolle de et al., 1999), little work has been carried out into the immunological response of the skin model to infection. Hence, although we cannot mimic the humoral *in vivo* immune response, we can easily investigate some of the cellular response of the skin equivalent to infection. Macrophages and granulocytes participate in the amplification of the cytokine network of the skin, while LCs and dermal dendritic cells present antigen to activated T-cells (Figure 2). The role of MCs, LCs and KCs in the SIS will be discussed.

Table 2: Cellular components of the SIS. Table modified from (De Bos and Kapsenberg, 1993).

CELLULAR CONSTITUENTS OF THE SKIN IMMUNE RESPONSE	
INNATE	ADAPTIVE
Tissue Macrophage	Langerhans cell
Monocyte/ Granulocyte	Dermal dendritic cells
Keratinocyte	T-cells
Mast-cells	Endothelial cells

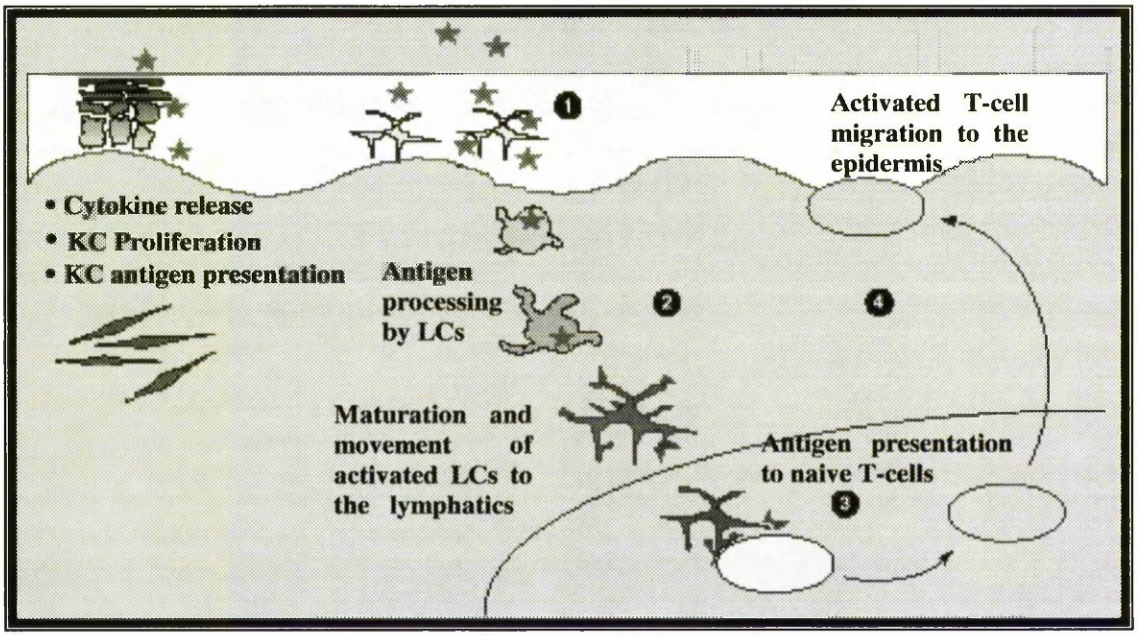


Figure 2: Cellular components of the skin immune response. Figure modified from (Grabbe and Schwarz, 1998)

1.3.5 KERATINOCYTES

KCs are one of the major immune cells of the epidermis capable of initiating a cytokine response which has a myriad of effects on the cells of the SIS. Culture of these cells on a dermal equivalent results in a comparable differentiation and stratification of the KCs into basal, spinous, granular and stratum corneum layers.The main KC function in the SIS is in the production of cytokines which follow a microbial/inflammatory challenge. Following direct exposure of certain ligands to the epidermis, KC respond with IL-1α release and this in turn effects the epidermis in several ways including increased proliferation and further cyokine production.

1.3.5.1 KC ANTIGEN PRESENTATION

KC are considered the non-profesional antigen presenting cells of the epidermis. As KCs express the MHC class HLA-DR protein it is thought that antigen can be expressed in association with this molecule. Expression of HLA-DR associated antigen results in upregulation of further HLA-DR expression, attracting T cells to the site of inflammation (Myint et al, 2000). While KCs do express HLA-DR constitutively, γ IFN has been shown to upregulate expression of this molecule on KCs (Basham et al, 1985) and while KC can produce γ IFN (Howie et al, 1996) it is thought that the recruitment of T cells to the site of antigen expression amplifies this expression as T cells express γ IFN. In terms of infection no evidence as yet exists as to the ability of KCs to directly process microbial or fungal antigen. While KCs do possess some phagocytotic ability (Luger et al, 1981), this has not been demonstrated in relation to fungal particles. Viral components, which do not require KC processing are however thought to be presented in association with HLA-DR molecules (Londei et al, 1984). Therefore as KC possess antigen presenting molecules they may amplify the initial immune response of the skin to infection by fungal agents.

1.3.5.2 CYTOKINES

KCs function *in vivo* on the skin as the main source of cytokines in an inflammatory response following trauma or infection (See table 3). The production of cytokines in the LSE has been measured in response to infectious agents (Fucso et al., 1993) and UV (Corsini et al., 1997), and has shown an equivalent response, due mostly to KC-derived cytokines. The innate immune response function of KCs *in vitro* seems to be equivalent to the *in vivo* profile. Aside from their immense cytokine profile, it has only recently been established that KCs also secrete complement component C3, a precursor for C3d and C3dg required for the activation of the alternative pathway of complement stimulation (Seguin et al., 1997). KCs also produce an anti-leukoproteinase which is a soluble serine protease inhibitor and one of the innate antimicrobial agents of human skin (Wiedow et al., 1998). This inhibitor directly acts as an antimicrobial agent by inhibiting proteolysis, contributing further to the innate SIS. Non-specific stimulation of the KC results in expression and release of these factors that act as primary immunological agents in the initiation and amplification of several non-specific and specific responses.

1.3.5.3 DEFENSINS

Antimicrobial peptides are an integral component of the innate immune system of many species of plants and animals (Boman, 1995). KCs produce HBD-2 and HBD-3 in response to inflammatory mediators, TNFα and killed whole bacterial and fungal cells (Harder et al., 1997). However the exact role of defensin in the SIS has not been fully elucidated. Over 70 defensin peptides are known, all sharing close structural and sequence homology, and five distinct types of peptide can be characterised; these will be discussed in chapter 2 in more detail. Those peptides with two or more disulphide bonds, commonly β-sheet structures and anti-parallel chains, are called β-defensins. These peptides are on average 29-34 amino acid residues in length, contain four or more interlinked half cysteines and are very basic with a high arginine residue content (Ganz and Lehrer, 1994). They are genetically coded as propeptides and are modified on secretion from cellular vacuoles into the active peptide. The common action of these defensins is on the membrane integrity of the organism (Lehrer et al., 1989). Primary electrostatic interactions facilitate the attachment of the peptides (cationic) to the organism's membrane (anionic), and heterodimerisation of the peptides results in pore formation and cell lysis. Human defensins have been mapped to chromosome 8, band 23, and they are found to be constitutively expressed in many mucosal sites; upregulation of transcription is found in states of infection/inflammation (Ganz and Lehrer, 1995). This has been linked to the presence of a NFκB (heterodimer) binding site upstream of the defensin gene and the presence of this binding site, and an IL-6 CTGGGA responsiveness binding site, point towards transcriptional upregulation of human β defensins in response to inflammatory mediators. The role of HBD-2 in protecting the skin from microbial challenge will be discussed in chapter 2 in more detail.

Table 3: Cytokine production by KCs.

CYTOKINES PRODUCED BY KERATINOCYTES	
IL-1α	IL-1 α accumulation in the KC is released by non-specific trauma to the skin. This cytokine in turn stimulates further cytokine production by KCs.
IL-6	Produced by KCs following IL-1α paracrine stimulation. Functions in B- and T-cell proliferation and differentiation and is an endogenous pyrogen.
IL-10	Increased transcription in response to cytokines, LPS etc. Stimulates IL-1ra production limiting IL-1α effects. Is chemotactic for monocytes, neutrophils and T-cells.
COLONY STIMULATING FACTORS (CSF)	GM-CSF, G-CSF, M-CSF and IL-3 which induce stem cell proliferation. LPS and IL-1α increase KC CSF production.
TNF α	Transcription is initiated by UV, cytokines and tumor promoters. Crucial for LC viability it stimulates NFκB transcription.
ENDOTHELIN-1	Produced by KC and endothelial cells in response to cytokine, stimulates αMSH production and vasoconstriction.
IL-8	Produced in response to IL-1α, a chemokine which acts as a chemoattractant for neutrophils.

1.3.6 LANGERHANS CELL

These dendritic cells are the main antigen-presenting cells of the skin and, until recently, have not been applied in skin models. By immunohistochemical analysis of skin it has been revealed that the main area of residence of LCs is the basal layer of the epidermis with dendritic extensions of the cells reaching into the stratum corneum. LCs are the main professional antigen presenting cell of the skin and interact with exogenous antigens, presenting them to the appropriate T cell population.

1.3.6.1 ANTIGEN PROCESSING AND PRESENTATION

Following skin exposure to antigen, LCs express maturation cell markers and in response to changes in their cell adhesion molecule expression and lymphatic and epidermal factors, they migrate to peripheral lymphatic organs (Weinlich et al, 1999). It is thought that LC movement from the epidermis occurs as the antigen is being processed (Axelrod et al, 1994). Once activated into maturation following antigen internalisation, the LC migrates through the epidermal/dermal junction, dermal perivascular unit and to the peripheral draining lymph nodes, where it stimulates T-cell populations by presentation of antigen in association with MHC complex. Migration is in response to both efferent lymphatic and skin-derived factors, and as such, LC antigen presentation is essential for the stimulation of the adaptive immune response of the skin (Kripke et al, 1990).

1.3.7 MELANOCYTES

MCs are found at low frequency in the normal epidermis and are the resident pigment-producing cells of the epidermis. The major function of MCs is the production of melanosomes, the pigment-producing centres of the skin, which are transported to KCs by secretion and endocytosis. These melanosomes then function in the production of melanin which protects cellular DNA from UV irradiation damage. The MC is also essential in amplifying the immune response of the skin, as it has recently been shown that these cells are capable of transcribing various cytokines in response to stimuli, including TGF-α, EGF and TGF-β (Morelli, 1993). MC control is exerted in part by KCs which have recently been shown to secrete pro-propiomelanocortin, a precursor of α-MC stimulating hormone (αMSH) and KC-derived IL-1α production stimulates αMSH receptor expression on MCs (Schauer et al., 1994).

1.3.8 ENDOTHELIAL CELLS

Endothelial cells act as important regulators of cell trafficking throughout the body. As these cells line the vasculature of the body they have a myriad of functions on many cell types including smooth muscle cells, where they can regulate growth and on fibroblast growth, as they secrete FGF. Most importantly endothelial cells regulate the transendothelial movement of material and cells between blood and interstitial fluid, thus regulating immune cell traffic. As one of the main components of the perivascular unit this is particularly relevant in the regulation of immune cells into and out of the epidermis. Thus in response to an immune signal, often cytokine production, endothelial cells express cell adhesion molecules which act as a chemoattractant for immune cells and this process has been termed 'Endothelial cell activation'(Pober, 1988). Signals from the epidermis stimulate adhesion molecule expression and ICAM-1, MHC class 1 and VCAM-1 have all been shown to be upregulated in response to inflammatory mediators (Schneemann et al, 1993). Expression of these receptor encourage B and T lymphocyte migration to the affected area. With particular reference to fungi, dissemination of *C. albicans* results in endothelial cell phagocytosis of the organism and increased ICAM-1 and VCAM-1 expression (Orozco et al, 2000). In skin, endothelial cells react mainly to the immunomodulating cytokines produced by the resident dermal and epidermal cells. It has been suggested that endothelial cells may themselves act as antigen presenting cells as they express HLA-DR (Hirschberg et al, 1980). However this has been shown to be unlikely, with LC thought to account for the vast majority of dermal antigen presentating cells of the skin.

1.4 AIMS AND OBJECTIVES

The aim of this work was to create and assess a series of skin models which would yield definitive answers as to *M. furfur*'s role in the various cutaneous diseases. The major questions to be addressed, regarding *M. furfur* infection were:

1. **Does *M. furfur* initiate an innate immune response?** The possibility of host-specific reactions to *M. furfur* in donor tissue might account for the reversion from commensal to pathogen in only some individuals.
2. **Do *M. furfur* or *M. furfur*-derived products directly alter melanin synthesis?** Clarification of the effects of *M. furfur* on melanin synthesis might directly implicate the organism in the pigmentory changes associated with PV.
3. **Does *M. furfur* grow at different rates on different donor tissue and in different skin models?** Differences in the growth patterns of the organism on different host tissues might account for the pathogenic nature of *M. furfur* in some individuals only.

2 ANTIMICROBIAL PEPTIDES

2.1 INTRODUCTION

The innate immune response of multicellular organisms provides a rapid, specific and effective defence against possible invading pathogens. Whereas clonal expansion of antigen-specific B and T-cells is a lengthy process, the innate immunity, and its multitude of constituents, provide an immediate barrier against the array of environmental pathogens encountered by the host. Antimicrobial proteins, first recognised in the form of lysozyme (Fleming, 1922), are some of the main constituents of innate immunity in plants, amphibians and mammals (see Table 4). These molecules represent one of the principal lines of non-specific innate defence and in particular the smaller antimicrobial peptides, classified as those less than 100 amino acids in length, are common throughout nature. The smaller antimicrobial peptides show common structure, function, sequence and transcriptional regulation indicating either a functional convergence or evolutionary divergence for these molecules. In light of the recent discoveries in Drosophila (Hoffmann, 1995) the theory of an evolutionary divergence has been widely accepted. The antimicrobial peptides remain a common and important constituent of the innate immune response of many organisms and they are an ancient component of this protective response. The smaller cationic peptides are a significant element of this non-adaptive immune response and they are ubiquitously found in a variety of epithelial secretions and in specific cellular compartments.

Table 4: Antimicrobial protein components of the human innate immune response.

PROTEIN	LOCATION	ACTION
Transferrin	Phagosomes	Competes for Fe^{2+} with the organism, inhibiting growth.
Defensin	Epithelial cells	Pore formation in organism's membrane resulting in cell lysis.
SLPI	Keratinocytes	Proteinase inhibitor which protects elastin fibers from proteolytic damage and is directly antibacterial.
Properdin	Plasma	Activates complement synthesis in absence of antigen/antibody stimulation.
Lysozyme	Phagosomes	Disrupts cell wall linkage resulting in cell lysis. *

* Disrupts N-acetylmuramic acid and N-acetylglucosamine linkage.

2.1.1 GENERAL FEATURES

The majority of information amassed on antimicrobial peptides is based on research carried out in insect systems (Boman, 1991). It was observed early in the 1980s that injection of the giant silkworm *Hyalophora cecropin* haemolymph with bacteria resulted in phagocytosis and clearance of the organism with a resultant overall increase in both RNA and protein synthesis (Steiner et al., 1981). The 9 proteins which were isolated from this moth were later found to have structural homology with each other and were subsequently termed the Cecropins. Much of the information regarding mammalian antimicrobial peptides has been generated in line with the development in knowledge of the Cecropins. Over the years the antimicrobial peptides, with now over 400 known, have been classified into 4 overall groups as determined by sequence, functional and structural homology (Boman, 1995).

2.1.1.1 LINEAR HELICAL PEPTIDES WITHOUT CYS

This grouping is composed mainly of the insect-derived molecules, particularly the Cecropins. These peptides have a strong basic N-terminus with a long hydrophobic stretch at the C-terminus. They have a 12 amino acid consensus sequence that is virtually unchanged throughout and are approximately 40-70 amino acids long.

2.1.1.2 LINEAR PEPTIDES WITHOUT CYS AND A HIGH PRO AND ARG CONTENT

The first peptide to be isolated with this structure was an apidaecin from the honey bee (Casteels et al., 1989). These peptides incorporate the bovine neutrophil peptides Bac 5 and Bac 7 and include the Drosophila drosocin peptide (Frank et al., 1990).

2.1.1.3 PEPTIDES WITH ONE DISULPHIDE BOND

This group includes bactenecin, isolated from bovine neutrophils (Romeo et al., 1988) and several antimicrobial amphibian skin peptides (Clark et al., 1994). These molecules are short, loop-forming peptides with 1 disulphide bond, often located at the C-terminus.

2.1.1.4 PEPTIDES WITH TWO OR MORE DISULPHIDE BONDS

This group contains the most biologically important class of mammalian antimicrobial peptides, the defensins. They contain six invariant cys residues and are basic peptides which form β-sheet structures with anti-parallel chains.

2.1.2 DEFENSINS

Defensins are the best studied family of what have been termed antibiotic peptides. Human defensins were first recognised in 1966 having been purified from phagocytic granules of neutrophils (Zeya and Spitznagel, 1966). The isolated peptide was subsequently recognised as a major component of the non-oxidative killing mechanism of neutrophils which is required to kill bacteria, and human neutrophil peptide 1 (HNP-1) was the first defensin ever formally classified (Selsted et al., 1983). By studying the innate immune mechanisms of neutrophils, and comparing this to the immunity of other insect and mammalian species, much progress has occurred in our understanding of defensins. These cationic peptides are produced by proteolytic cleavage of a 29 amino acid precursor and are antimicrobial for a range of bacteria, fungi and enveloped viruses. They are produced by a variety of species and are abundant in nature. They include:

Insect: Drosomycin is produced by the liver-like fat body of the insect and is extremely active against the fungal pathogens of Drososphila (Meister et al., 1997).

Plant: Inducible production of *Raphanus sativus* antifungal peptide is initiated in the plant *Raphanus sativus* when the radicle places pressure on the outer lamellar layer of seed. This is thought to protect the germinating seed from initial fungal infection (Tailor et al., 1997).

Mammalian: Tracheal antimicrobial peptide (TAP) and lingual antimicrobial peptide (LAP) of bovine origin are produced in response to infection and inflammation of the cows trachea and tongue respectively (Diamond et al., 1991).

2.1.3 HUMAN DEFENSINS

With particular reference to humans there are only 8 defensins presently identified.

HNP1-4: Human neutrophil peptide is isolated from the auzorophil granules of neutrophils and accounts for 85% of the protein content of neutrophils. These peptides are stored in the specialist auzorophil granules and act when bacteria are phagocytosed. They function by initiating pathogen cell permeabilisation.

HNP5-6: These molecules are produced by Paneth cells of the small intestine, and are secreted into the intestinal villi crypt. They are also referred to as human antimicrobial defensin 5 and 6 (HAD5-6).

HBD-1: Human β defensin-1 is secreted mainly in the genito-urinary epithelia and is also isolated from trachea and conjunctiva.

HBD-2: HBD-2 is found in skin and pulmonary epithelia and is released from the cells in

response to inflammatory stimuli.

The human defensins can be further classified into two major groups, α and β, as determined by their structure.

2.1.4 α DEFENSINS

These peptides have 6 invariant cys residues and hence 3 disulphide bonds at C1-6, C2-4 and C3-5. They are synthesised in a 90 amino acid precursor form, which becomes an active peptide of approximately 29-30 amino acid length. X-ray crystallography of the HNP-1 homologue, rabbit NP-1, has shown its 3D secondary structure to be composed of a triple stranded beta sheet with connecting hairpin loop. This group contains HNP1-3 and the intestinal defensins HNP5-6 (Hill et al., 1991).

2.1.5 β DEFENSINS

These peptides are slightly longer, 38-47 amino acids long, with high Arg and Lys residue content. Disulphide bonding occurs at C1-5, C2-4 and C3-5 with dimerisation of the β-defensins occurring at the opposing hairpins. This results in a 4-stranded hydrophobic β-sheet and these peptides are expressed in a range of animals. HBD-1 and HBD-2 belong to this β defensin classification (Table 5).

2.1.6 HUMAN β DEFENSIN-1

HBD-1 was first isolated in 1995 when experimentation to improve the extraction of peptides from human haemofiltrate revealed a novel molecule with extensive homology to the bovine β defensins (Bensch et al., 1995). This peptide was later named human β defensin 1 (HBD-1) and has the characteristic 6 Cys repeat sequence common to human, bovine and other mammalian defensins.

2.1.6.1 SITES OF PRODUCTION

Purification of the peptide from vaginal lavage fluid has permitted sequencing and cloning of the gene, allowing construction of mRNA probes and monoclonal antibodies specific for the peptide to measure expression of transcription and effective translation of HBD-1 in tissues. Following these experiments it has been established that the peptide is produced by a range of epithelial tissues including the respiratory tract (Zhao et al., 1996), urogenital epithelia (Zhao et al., 1996), ocular epithelia (Haynes et al., 1999), placenta (Svinarich et

al., 1997) and intestinal epithelia (O'Neil et al., 1999). At least sixteen isoforms of the peptide, ranging from 36-47 amino acids in length, have been shown to be produced by the genito-urinary epithelia (Hiratsuka et al., 2000).

Table 5: β-Defensins from various animal species.

NAME OF PEPTIDE	ANIMAL	TISSUE EXPRESSED
TAP/LAP: Tracheal and Lingual antimicrobial peptide	Cow	Trachea & tongue epithelia.
SBD-1, 2: Sheep β defensin-1,2	Sheep	Gastrointestinal tract.
HBD-1: Human β defensin-1	Human	Kidney, salivary gland & trachea.
HBD-2: Human β defensin-2	Human	Skin.
Gallinacins	Chicken	Heterophils.
THP 1-3; Turkey heterophil peptide-1-3	Turkey	Heterophils.
PBD-1: Porcine β defensin-1	Pig	Tongue, respiratory and gastrointestinal tract.

2.1.6.2 ROLE IN DISEASE

Using semi-quantitative RT-PCR and *in situ* hybridisation/immunohistochemistry, it was possible to establish that there is no upregulation of HBD-1 transcription in response to inflammatory stimuli in most infection models (Zhao et al., 1996). In nude mice models it has been shown that the antimicrobial effects of bronchial fluid are due to HBD-1, as treatment of the cells with antisense HBD-1 results in loss of the bacteriostatic activity of the fluid (McCray and Bentley, 1997). These results indicate that this defensin, in common with many antimicrobial peptides, has a role in maintaining the innate immunity of the epithelia from which they are produced. In addition there is a putative connection between a lack of HBD-1 production in some malignancies. It has been proposed, following a study

on the lack of HBD-1 production in selected carcinoma epithelial cell lines, that lack of control of the microflora and subsequent establishment of a damaging infection may be responsible for conversion to malignancy (Abiko et al., 1999). This is the hypothesis linking *Helicobacter pylori* infection to intestinal cancer and hence warrants further investigation particularly in oral carcinoma. Finally the production of HBD-1 in mammary gland epithelia supports the hypothesis that this peptide may also be essential in neonatal immunity via colostrum (Tunzi et al., 2000).

2.1.6.3 GENETIC INFORMATION

Production of cDNA allowed cloning and isolation of the HBD-1 gene on human chromosome 8, band p23 (Figure 3). This locus is 150bp away from the α-defensin human neutrophil genes indicating common phylogenic ancestry between these defensins, despite their structural and sequence differences.

2.1.7 HUMAN β DEFENSIN-2

HBD-2 (Figure 4) was first isolated in 1997 following investigation into the infrequency of cutaneous infections in psoriatics (Harder et al., 1997). On dissolution of psoriatic scales, this peptide was isolated and purified and shown to have sequence similarity to bovine LAP and TAP. In common with its homologues, HBD-2 is produced by lung, kidney, stomach and small intestine at a basal level, as shown by both Northern blotting and RT-PCR (Bals et al., 1998).

2.1.7.1 SITES OF PRODUCTION

HBD-2 mRNA and peptide production has been detected in many epithelial cells including lung gland and pulmonary epithelia (Becker et al., 2000). Basal expression of HBD-2 is found primarily in skin, lung, trachea, and salivary gland epithelia (Singh et al., 1998; Weinberg et al., 1998).

2.1.7.2 GENETIC INFORMATION

Isolation of the HBD-2 gene reveals its location on chromosome 8, region 8p21. HBD-1 and HBD-2 share the same 1.5MB region; hence, this area is thought to be responsible for the coding of human β-defensins. Drawing comparisons from the work accumulated on bovine and porcine β-defensin expression, HBD-2 was thought to be induced in a similar

way by inflammatory stimuli. Several lines of experimental data support this theory:

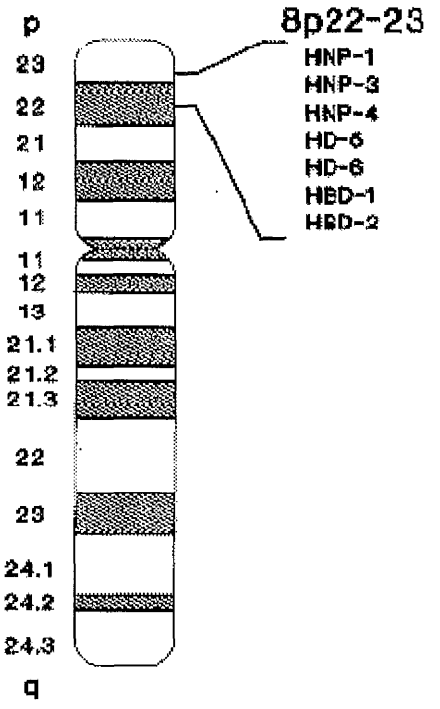


Figure 3: Diagram of chromatin fiber FISH mapping of human defensin genes (Liu et al., 1998).

***In vivo* models.** HBD-2 expression has been shown *in situ* in wounded skin (Ali, 2000). In addition, lung epithelia expression is increased as measured in bronchioalveolar lavage fluid (BAL) in response to pneumonia (Hiratsuka et al., 1999).

***In vitro* models.** In addition to TAP and LAP experimental models, human tracheal epithelial cells are shown to up-regulate HBD-2 expression in response to TNFα, IL-1 β and LPS (Becker et al., 2000).

NFκB consensus sequence. Sequence data from the TAP and LAP defensins show an IL-6-responsive element upstream of the 5'flanking region. This sequence is not evident in HBD-2, and *in vitro* experimental data show that the gene is not induced by the exogenous addition of IL-6. There are however 4 NFκB-binding sites associated with the gene, one approximately 208 base pairs upstream of the TATA box region, indicating inflammatory stimuli as promoters. (Diamond et al., 1991).

Toll-like receptors (TLRs). Having observed the stimulatory capacity of IL-1β, it was

assumed that the TLR, IL-1R1, was involved in transduction of the signal for HBD-2 activation. Binding of the TLRs in association with receptor-associated molecules, thought to be CD14 in humans, results in receptor-associated kinase recruitment and phosphorylation of the kappa kinase subunits. This in turn results in proteolytic destruction of the Iκβα subunit, NFκB inhibitor, which then allows NFκB to advance to the nuclei and bind to the NFκB consensus sequences. Recent experimentation on this theory supports the association of CD14 and TLRs, or as they are also known, pathogen pattern recognition receptors (PPRRs), in the activation of NFκB. By producing a monoclonal antibody for CD14, *in vitro* upregulation of HBD-2 transcription was blocked in an experimental bronchial model (Becker et al., 2000). Although limited evidence exists for this PPRRs selective interaction in humans, in Drosophila it has been shown that drosomycin expression is selectively expressed in response to fungal challenge, but not gram-negative bacteria. This suggests a discriminatory response to pathogens by inducible-specific peptides, and hence a selectivity by the Toll receptors in induction of these genes (Lemaitre et al., 1997).

2.1.7.3 ROLE IN DISEASE

The production of defensins and antimicrobial peptides in the mucosa of animals is thought to prevent the establishment of infection by controlling commensal colonisation and preventing infection by exogenous microorganisms. To test this hypothesis it had to be established whether there was any change in expression of defensins at sites of infection. Alternatively any loss in activity or expression of antimicrobial peptides might also result in faster or more aggressive disease progression.

2.1.7.4 UPREGULATION IN INFECTION

The upregulation in transduction of the β-defensins in infection has been demonstrated in the bovine β defensins for some time (Schonwetter et al., 1995). Isolation of LAP from bovine tongue epithelia allowed the measurement of β-defensin expression from the tongue epithelia in cases of chronic infection, indicating the dramatic increase in β defensin expression in the infected tissue. In humans, Harder et al (1997) were able to quantitate the increase in expression of HBD-2 in psoriatic skin and demonstrated the inducibility of this peptide in response to the inflammatory stimuli, LPS and TNF-α. HBD-2 upregulation was

also detected *in vivo* in patients suffering from bacterial pneumonia (Ashitani et al., 1998).

2.1.7.5 INACTIVATION OF PEPTIDES

One of the most interesting pathological consequences of defensin inactivation occurs in cystic fibrosis where the establishment of chronic pulmonary infections occurs in patients. Mutational defects in the gene transcribing the cystic fibrosis transmembrane conductance regulator results in a reduction in the effectiveness and distribution of this channel on the pulmonary epithelia. The consequence of this abnormality is an alteration in the ionic composition of the surface airway fluid. The exact measurement of the ionic composition of airway surface liquid has been fraught with difficulty and is greatly disputed; however, there is a general consensus that the concentration of Cl^- is higher in the airway surface liquid of cystic fibrosis patients (Knowles et al., 1997; Olson et al., 1996). Both HBD-1 and HBD-2 have been shown to be 'salt-sensitive' at the physiological levels proposed in cystic fibrosis and in particular one of the main respiratory pathogens of cystic fibrosis patients, *B. burgdorferi*, is resistant to the action of defensin at these higher salt concentrations. In another inactivation model, the creation of a drosomycin knockout in Drosophila resulted in a fatal pulmonary infection indicating how important this peptide is in respiratory defence of Drosophila (Meister et al., 1997).

2.1.8 FUNCTION AND STRUCTURE OF ANTIMICROBIAL PEPTIDES

2.1.8.1 FUNCTION

Defensins exert various effects on mammalian, bacterial and fungal cells. The main putative consequence of defensin expression is its selective permeabilisation of bacterial and fungal cell membranes, however several other effects that are thought to assist in immunity and healing have been investigated.

***Chemoattractant*:** Several experiments have demonstrated the chemoattractant capabilities of defensins. Cryptidin, from Paneth cells, is a chemoattractant for chemokine receptor six (CCR6)-expressing cells (Wilson et al., 1999). These cells include memory T-cells, LCs and CD34+ cells, and these are shown to migrate *in situ* in response to a cryptidin gradient. This indicates a role for defensin in linking innate and adaptive immune responses when in states of infection; the chemoattractive concentration gradient of defensin produced at the site of infection attracts a cellular response. In this respect the innate immune response

(defensin) links the adaptive (T-cell and LC) immune responses together; in addition defensin is also shown to bind the C1 component of complement (Panyutich et al., 1994).

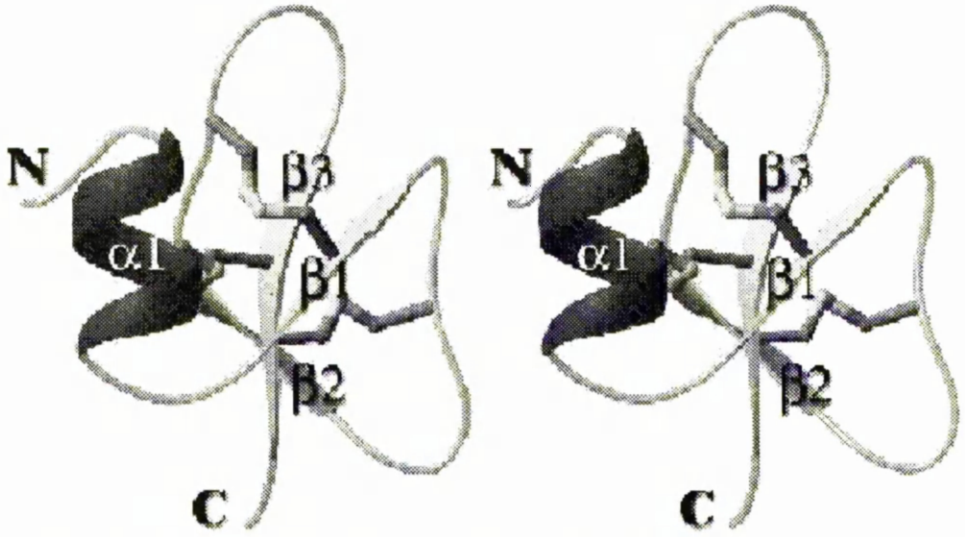


Figure 4: Stereo drawing of the dimerisation of HBD-2 (Hoover et al., 2000).

Anti-tumour: When a magainin-expressing construct was transfected into mouse bladder carcinoma cells, tumourogenicity, as measured by tumour size, was greatly reduced (Winder et al., 1998). In addition, Cecropins have been shown to lyse both leukaemia and lymphoma cell lines (Moore et al., 1994). Although both of these experiments are examples of insect defensin acting against animal tumourogenic cell lines, some evidence exists of an immunosurveillance role in human cancer for defensins. HNP-1 is expressed in the duct cells of the submandibular glands of oral cancer patients, detected by immunohistochemistry (Mizukawa et al., 2000). In addition, there is a significant increase in the concentration of HNP-1 isolated from the saliva of oral squamous cell carcinoma patients, as measured by HPLC (Mizukawa et al., 1998) and a decline in HBD-2 expression (Abiko et al., 1999). This rise in squamous cell carcinoma HNP-1 expression coupled with a decline in HBD-2 expression may be responsible for some of the pathology associated with the tumours or it may be a by-product of the cellular disruption associated with the metabolism of the tumour cells.

Mitogenic: There is sparse evidence for the mitogenic properties of defensin, but DNA synthesis assays show the stimulatory effects of Human neutrophil defensins 1-3 on epithelial cell lines and fibroblasts (Murphy et al., 1993). This supports the evidence that

purified mouse defensin promotes the healing of aseptic wounds by exerting a 'growth factor-like' effect (Gallo et al., 1994).

Corticostatic: The N-terminal regions of some defensin peptides inhibit rat adrenocortin. The adrenocorticotropic hormone ACTH releases cortisol, an immunosuppressive molecule on binding of this ligand to its adrenocortin receptor. Defensins are thought to compete with ACTH, so preventing the release of this immunoinhibitory peptide (Zhu et al., 1988). The role therefore of antimicrobial peptides in the innate immune response is not solely confined to protective mucosal and neutrophil immunity.

Supplementation of immune response: Defensins exert a greater-than-accumulative effect with lysozyme in the killing of Gram- negative bacteria. This positive co-operation is greater than the sum of the bacteriolytic effect of each individually (Hancock and Scott, 2000). In addition, HNPs enhance both antigen-specific IgG responses and T-cell proliferation in mouse models, as compared to antigen alone (Lillard et al., 1999).

DNA control: In contrast to the mitogenic effects as previously discussed, defensins have been shown to act in an immunosuppressive manner. In LPS-induced sepsis, cytokine release from macrophages occurs and cationic peptides inhibit this reaction by suppressing both TNF-α and IL-6 production. Hence these peptides act in a negative feedback loop, controlling and limiting the damage of infection as well as attempting to directly control the growth of infectious agents (Scott et al., 1999). In support of this theory it has been recently demonstrated that the cathelicidin-like peptide PR-39 directly interacts with the NFκB inhibitor IκBα, directly preventing NFκB activation (Gao et al., 2000). As mentioned previously, NFκB is instrumental in the transcription of many inflammatory agents, and by binding to this inhibitor this peptide prevents transcription of the NFκB-sensitive genes (Tsutsumi-Ishii et al., 2000). Defensins have also been shown to interact with DNA backbone, inducing single strand breaks (Gera and Lichtenstein, 1991).

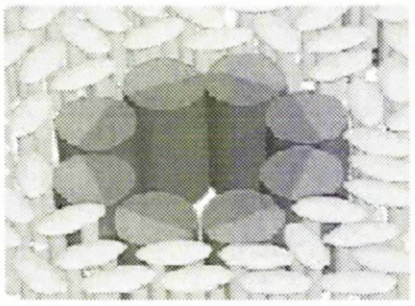
2.1.8.2 MEMBRANE PERMEABILISATION

The most widely examined action of defensins involves their ability to initiate membrane permeabilisation of microorganisms. Much of the work investigating membrane-antimicrobial peptide interactions has utilised the lipid bilayer model, and this has allowed

the elucidation of several theories on the function and action of these peptides on bacterial and fungal membranes (Vaz Gomes et al., 1993). Some of the first experiments carried out showed the need for a charged target membrane; hence a biologically active microorganism is required. It is thought that this charged membrane and electromotive force allows initial electrostatic interactions that constitute the first binding stage of the peptide to the target membrane. In this way alone defensins would specifically target negatively charged pathogen membranes; as most eukaryotic cells have a positive membrane thus the defensin cannot select 'self' for destruction. By producing synthetic chiral peptides and assaying their antimicrobial activity, it was been shown that there is no difference between the D and L enantomers (of magainin) indicating a non-stereospecific binding; therefore the primary electrostatic interactions may account for a large proportion of the binding process. Defensins act against the inner membrane of Gram-negative bacteria since disruption of the outer membrane with mannitol produces no change in their bacteriolytic capacity (Lehrer et al., 1989).

In lipid bilayers, HNP-1 produces voltage-dependent, ion-permeable channels and this interaction is dependent on the charge of the peptide (Kagan et al., 1990). As the human defensins also contain a 10 amino acid, anti-parallel β sheet that is homologous to the porins, it is thought that, like the porins, they dimerise in the target membrane forming a pore. Recently groups from Texas (Huang, 2000) and Koto (K. Matsuzaki 1998) have proposed a 'Barrel stave' model (Figure 5), which combines many of the known aspects of antimicrobial peptide interaction with membranes. The Barrel stave model suggests an initial binding to the membrane, due to cationic (antimicrobial peptide) and anionic (bacterial membrane) interactions. The α-helix of the peptide is then thought to lie parallel to the target membrane, with heterodimerisation and insertion into the membrane occurring. At least 5 α-helices and several lipids bond to form a pore and the resultant ion transport produces fatal cell permeabilisation and cell death. Ion-permeable pores are produced by the α-defensins and in particular HNP-3, which forms a 20Å membrane-spanning pore when 12 monomers aggregate. However another model termed 'the carpet model' also exists. In this model the peptides are thought to interact with the anionic lipid headgroups of the target membrane, thus neutralising a distinct area and disrupting overall ion transport. Recent elucidation of the quaternary structure of HBD-2 suggests that this is the mechanism of action for this peptide (Hoover et al., 2000).

A



B

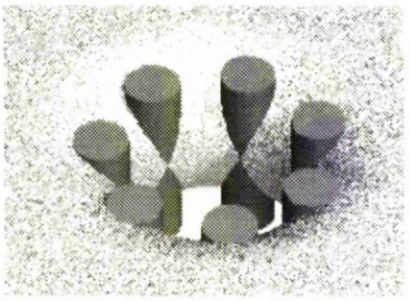


Figure 5: Barrel stave model figures for the formation of magainin pores (Ludtke et al., 1996).

A/ Monomers hydrophobic and hydrophilic regions represented by the red and blue region of the cylinder respectively.

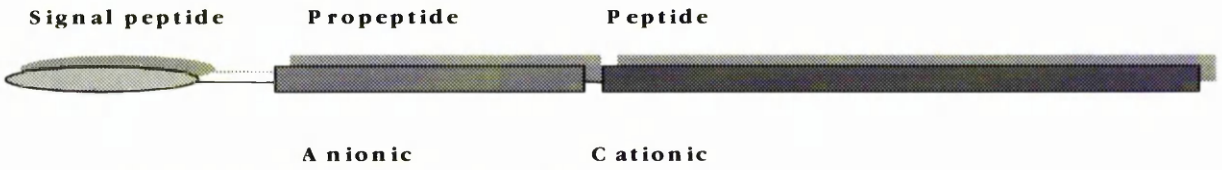
B/ Magainin monomers embedded in the lipid headgroup region (Huang, 2000).

2.1.8.3 STRUCTURE

Analysis of the cDNA sequences of the various isoforms of HBD-1 isolated from urine indicates that defensins are translated as propeptides and subsequently enzymatically activated (Hiratsuka et al., 2000). Analysis of the 16 isoforms of HBD-1 shows the same 'active' C-terminal with differing N-terminal sequences (Figure 6).

These peptides are thought to be produced as a 68 amino acid prepropeptide which on loss of its signal peptide is a 47 amino acid pro-HBD-1 peptide. This peptide is then proteolytically cleaved into the mature 36 amino acid peptide. Further evidence exists for this post-translational cleavage mechanism of activation for antimicrobial peptides. The Paneth cell peptide cryptidin is co-localised in crypt cells with a matrix metalloproteinase (MMP), matrilysin. Procryptidin, the peptide isolated from the crypt cells, is not active until proteolytically cleaved by matrilysin (Wilson et al., 1999). In addition, transgenic mice that are MMP-deficient are not protected from intestinal infection by their Paneth cell peptides as they do not become activated in the absence of the MMPs. Tertiary structural analysis has been carried out on many of the animal defensins; however, the α-and β-defensins differ only in their location of disulphide bonding (Figure 7).

Figure 6: Diagrammatic representation of the inactive pre-propeptide that is post-



translationally modified to active form.

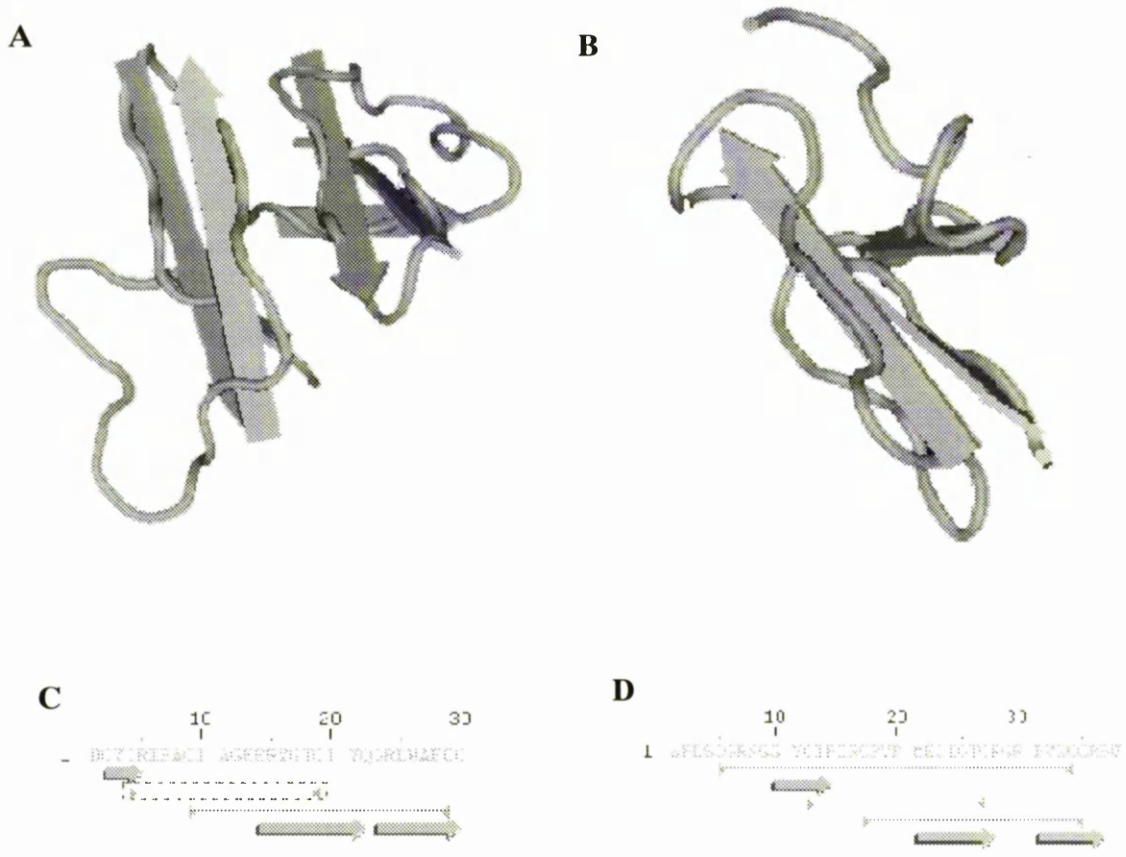


Figure 7: HNP-3 α defensin structure and bovine β defensin-2 structure

A/B: 3D structural representation of the HNP-3 **(A)** and BBD-2 **(B)** molecules

C/D: Sequence comparison of the α-helical and β-sheet domains of the α-defensin **(C)** and β-defensin **(D)**.

⟹ Depicts β sheet structural and sequence homology

⟹ Depicts α helical structural and sequence homology

Sequences and 3D peptide models obtained from the NCBI protein data bank web pages (http://www4.ncbi.nlm.nih.gov/PubMed), viewed using CD-3D programme.

2.1.9 ANTIMICROBIAL PEPTIDES AND SKIN

Two classes of peptide have been isolated from mammalian skin, all isolated in cases of

infection and inflammation.

2.1.9.1 CATHELICIDINS

Two types of peptide belonging to this family have been isolated from mammalian skin.

PR-39: This proline-rich peptide was first isolated from porcine intestine in response to infection. It is a basic molecule secreted as a pre-protein and post-translationally modified into a mature 39 amino acid (Agerberth et al., 1991). Although belonging to the cathelicidin family of peptides, on the basis of its pre-protein sequence, this is a novel molecule that is isolated from porcine wound fluid and induces proteoglycan synthesis including syndecan –1 and –4 (Gallo et al., 1994). It is thought that this peptide is produced in wounds by migrating neutrophils and as yet no localisation of production by KCs has been observed.

LL-37: This peptide is also termed human cationic peptide 18 (hCAP-18) and its mature form is a cys-free α-helical amphipathic peptide. It is produced as a pre-protein FAL-39 which belongs to the cathelicidin family, sharing the homologous N-terminal pro-region. This peptide is the only member of the cathelicidin family to be expressed in humans and its production has been observed in granulocytes, testes, wound fluid and bone marrow (Frohm et al., 1996). In contrast to PR-37, LL-37 production is localised to KCs, and in addition it is widely expressed in psoriasis and nickel allergies. IL-8 and acute-phase response factor binding sites have been mapped upstream of this gene, indicating inflammatory control of transcription (Frohm et al., 1997).

2.1.9.2 β DEFENSINS

Two β defensins have been identified in mammalian skin.

LAP: LAP shares substantial sequence homology with HBD-2 and is produced in bovine skin in response to experimental infection (Stolzenberg et al., 1997).

HBD-1 and 2: HBD-2 was initially isolated from psoriatic skin and is thought to contribute to the low incidence of cutaneous infection in psoriatics (Henseler and Christophers, 1995). The SIS is composed of a multitude of resident cellular and humoral components, and

HBD-2 is an inducible peptide associated with maintaining the skin's tegument. HBD-2 is expressed by KC monolayers in response to TNF-α, Gram-positive, Gram-negative bacterial cells and fungi (Harder et al., 1997). HBD-1 is constitutively produced in normal skin, localised to suprabasal KCs (Fulton et al., 1997). These data are consistent with recent findings that show constitutive production of HBD-1 in burn wounds with a lack of HBD-2 expression (Milner and Ortega, 1999). It is thought that the primary action of HBD-1 is to prevent colonisation of the skin by commensal bacteria, accounting for its constitutive transcription. In contrast, HBD-2 is induced in response to infection or wounding, and as yet, little information surrounding its role in infection control exists.

2.1.10 OBJECTIVES

The purpose of this work was threefold:

- To determine the levels of expression of HBD-2 in different skin models, ie. KC monolayers, LSE and BRT.
- To measure any changes in defensin expression in response to *in vitro* infection with *M. furfur*.
- To establish whether there are any donor differences in the HBD-2 response to the organism.

2.2 METHODS

2.2.1 TISSUE PREPARATION

2.2.1.1 LSE

Preparation of dermal equivalent: All tissue culture medium components were obtained from Life Technologies, Paisley, UK and all tissue culture plastics were obtained from Costar UK Ltd, The Valley Centre, High Wycombe, Bucks, UK. Type I collagen was extracted from rat tail tendons in 0.5M acetic acid, precipitated by the addition of an equal volume of 10% (w/v) NaCl, redissolved in 0.25M acetic acid and extensively dialysed against 1/1000 acetic acid, with the final solution being adjusted to 2.5mg collagen/ml. Collagen gels were prepared by mixing 8 volumes of collagen solution with 1 volume of 10x minimal essential medium (MEM), 1 volume of 0.22M NaOH; the pH was finely adjusted to 7.2 with NaOH. One volume of foetal calf serum (FCS) containing 7.5 x 10^5 human adult breast skin fibroblasts[1] /10ml of gel mixture was added, thoroughly mixed, and 4.0ml added to 35mm petri dishes. The gels were allowed to set at 37°C for 15min, after which 1ml of MEM supplemented with penicillin (100 units/ml), streptomycin (100ug/ml), L- glutamine (2mM) and 10% FCS was added, the gels detached from the dishes, and incubated for approximately 10 days. Cultures were incubated at 37°C in a humidified atmosphere of 5%CO_2 in air, with medium changes every 3 days. The gels at this stage are highly contracted, with the fibroblasts adopting a spindle-shaped morphology.

KC culture: Human juvenile foreskins, derived from donors between the ages of 2-5 yrs old undergoing routine circumcision, were cut into 3mm strips after removal of subcutaneous fat. These strips were then floated on to 0.5% (w/v) dispase (Sigma-Aldrich, Poole, Dorset, UK) in phosphate buffered saline (PBS) and the tissue incubated overnight at 4°C. The epidermis was peeled off with forceps, finely chopped with a scalpel, and treated with 0.25% (w/v) trypsin in PBS for 5min with pipetting to obtain a predominantly

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[1] Fibroblasts obtained from explant cultures of reduction mammoplasty skin from one 43yr old female Caucasian, and used between passages 4 - 9. Same fibroblasts used throughout.

single cell suspension. The cell suspension was passed through sterile gauze, centrifuged (4min at 400xg) with the cell pellet resuspended in Dulbecco's MEM supplemented with 10% FCS to give an inoculating suspension of 2 x 10^5 cells/ml. This was then added to the surface of contracted collagen lattices in 24-well multiwell dishes. Following incubation at 37°C for 5/6 days as submerged cultures in 5% CO_2, the gels were then raised to the air/liquid interface and incubated for at least a further 6 days, after which a substantial stratified epidermis was obtained with a well developed stratum corneum. 15 µl of microbial cell suspension was then added to the dorsal surface of the skin model.

2.2.1.2 EXCISED TISSUE

***Breast reduction tissue*:** BRT was obtained from consenting female donors undergoing breast reduction operations at Canniesburn Hospital, Bearsden, Glasgow. Following excision of tissue, within 24hr of surgery, subcutaneous fat was removed aseptically with a scalpel. The tissue was then cut into 1cm^2 pieces, mounted onto sintered glass discs in a 35mm dish with medium added to just below the epidermal surface (CO_2-independent MEM supplemented with 10% FCS). The specimens were then incubated at 37^0C in a humid atmosphere until inoculation, no more than four hours after preparation. 15 µl of test sample or organism was then added to the surface, incubated at 37^0C in a humid atmosphere and epidermal RNA isolated.

2.2.2 CELL CULTURE

2.2.2.1 KC MONOLAYERS

Foreskin KCs obtained from juvenile circumcisions, as detailed above, were seeded into 24-well multiwell dishes at approximately 2 x 10^5 cells/well. Cells were then incubated in MEM supplemented with 10% FCS, at 37°C, 5% CO_2 for 10-14 days (depending on the viability of the initial cell suspension) until they formed a standard monolayer. Monolayers were incubated with serum-free MEM for 48hr and washed with 1ml PBS prior to inoculation with test organism/ extract.

2.2.3 ORGANISM PREPARATION

2.2.3.1 M. FURFUR

A single strain of *M. furfur* was isolated from PV-positive microscopic skin scrapings received at the Regional Mycology Reference laboratory, Western Infirmary, Glasgow. Skin scrapings were inoculated into Dixon's medium[2] and incubated at 37^0C for 72hr. *M. furfur* 'Hook' isolate alone was used throughout and maintained as a stock lawn culture.

Viable organism: A loopful of *M. furfur* from a pure lawn culture was emulsified in 5ml PBS. 10mg of sterile glass beads were then added to the universal and this was shaken at 230rpm for 2hr. The resultant supernatant was removed, centrifuged (5000xg, 15min) and the cell pellet washed twice in PBS. The pellet was resuspended in MEM (KC monolayer experiments) or PBS (LSE, BRT experiments) and in a cell suspension of 1 x 10^4 cells/ml was obtained after counting cells in a haemocytometer.

Killed whole cells: A loopful of *M. furfur* from a pure lawn culture was emulsified in 5ml PBS, autoclaved (120^0C, 20min) and centrifuged (5000xg, 15min). The pellet was then washed twice in PBS and the killed whole cells resuspended in either MEM (KC inoculation) or PBS (LSE or BRT inoculation) to give a cell suspension of 1 x 10^4 cells/ml.

2.2.3.2 C. ALBICANS

A single strain of *C. albicans* was isolated from oral epithelial swabs received at the Regional Mycology Reference Laboratory, Western Infirmary. Swabs were spread over GPC[3] agar and grown at 37^0C for 24hr. This *C. albicans* isolate alone was used throughout and maintained as a stock lawn culture

Viable organism: A loopful of *C. albicans* from a stock culture on GPC agar was emulsified in 5ml PBS. The resultant cell suspension was centrifuged at 5000xg for 15min, the pellet washed twice in PBS and then resuspended in MEM (KC monolayer experiments) or PBS (LSE, BRT experiments) to give a cell suspension of 1 x 10^4 cells/ml.

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[2] Dixons agar: 26g malt extract, 6g peptone, 10g agar, 20g ox bile, 10ml tween (40), 2ml glycerol, 2ml oleic acid, 10ml chloramphenicol, 10ml actidione in 976ml H_2O autoclaved.

[3] GPC: 20g agar, 40g glucose, 10g peptone in 990ml of distilled water. Following autocalving 10ml chloramphenicol added and agar poured and set.

2.2.3.3 *CELL WALL PREPARATION*

M. furfur and *C. albicans* cell walls were prepared by staff in the Infection and Immunity Division of the University of Glasgow, Scotland. In brief, 500ml of Dixon's broth was inoculated with a loopful of organism taken from a stock culture (GPC agar for *C. albicans;* Dixon's agar for *M. furfur*). Following incubation at 37°C for 48hr the culture was centrifuged (5000xg, 5min) and the cell pellet washed three times in PBS (20ml). The final pellet was resuspended in PBS (10%, w/v) and added to 30 ml of Ballotini beads in a Braun bottle. Following 1hr incubation on ice, cells were disrupted in a Braun homogeniser. Following microscopic confirmation of cell breakage, the suspension was filtered and centrifuged (500xg, 5min) to deposit whole cells, and further centrifuged (15000xg, 20min) to isolate cell wall fragments. The cell wall pellet was then washed six times in PBS and three times in distilled H_2O, and was finally shell-frozen in a round-bottomed flask prior to freeze drying.

2.2.4 MOLECULAR METHODS

2.2.4.1 *RNA PREPARATION*

***LSE*:** By shearing the upper epidermal layer from the underlying collagen matrix, a full epidermis was isolated from the LSE. Homogenisation of this sheet was achieved by fine chopping with a scalpel blade and the chopped tissue was added to a DEPC[4]-treated Eppendorf (500µl DEPC in 50ml H_2O microwaved with tubes for 1hr). RNA was isolated by using a Qiagen 'RNAeasy' preparation kit (Qiagen, UK). In brief, 600µl of RLT lysis buffer (a highly denaturing guanidinium isothiocyanate-containing buffer with 10% β-mercaptoethanol) was added to the tissue and incubated at room temperature for 10min. Tissue was then homogenised by repeated passage of the buffer/tissue mixture through a 1000µl pipette tip (Gilson) until a clear thick suspension was obtained. The sample was further homogenised by adding the 600µl of homogenate to a QIAshredder column and centrifuged for 2min at 5000xg. One volume (600µl) of 100% ethanol was added to the sample which was then added to the RNAeasy column and centrifuged for 15s at 5000xg.

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[4] DEPC (Diethylpyrocarbonate)

The silica membrane in the column was washed once with buffer RPI (to remove debris) and twice with buffer RPE (to ethanol-precipitate the RNA). RNA was eluted by adding 50µl of DEPC-treated H_2O[5] to the membrane, incubating for 1min at room temperature and centrifuging the column for 2min 15000xg.

BRT: Epidermal RNA was isolated by cutting the tissue into 5 x 2mm strips. These strips were then floated on to 0.4% (w/v) dispase in PBS and the tissue incubated overnight at 4°C. The epidermis was peeled off with forceps, finely chopped with a scalpel, and treated with 0.25% (w/v) trypsin in PBS for 5min with pipetting to obtain a predominantly single cell suspension. The cell suspension was passed through sterile gauze, centrifuged (4min at 400xg) and the cell pellet was lysed in 600µl RLT buffer. Thereafter the isolation of BRT was identical to LSE RNA retrieval.

***KCs*:** Monolayers were washed in PBS (2ml) and thereafter 600µl of RLT buffer was added directly to each well of the multiwell dish. Cells were homogenised by repeated pipetting with a 1000µl Gilson until a clear lysate was achieved and this was added directly to the RNAeasy spin column after the addition of 1 volume of ethanol.

***RNA Integrity*:** 10µl of RNA was added to 5µl of loading buffer and visualised on a 1% 1xMOPS[6], RNAse-free mini gel run at 60 volts for 1hr.

RNA Quantity: RNA was quantified by adding 5-10µl of purified RNA to RNAse-free H_2O in a 0.5µm quartz cuvette. Purity of the sample was determined by the A_{260}/A_{280} ratio. In brief the wavelength was blanked at 260nm of the RNAse-free H_2O. An A_{260} value of 1 for H_2O plus RNA is equal to 44.19µg/ml of pure RNA. Hence;

A_{260} value x dilution factor x 44.19 = RNA µg/ml in sample.

***RNA purity*:** The purity of the RNA sample was determined both by PCR (PCR of RNA that was negative was considered to have no DNA contamination) and the A_{260}/A_{280} ratio

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[5] DEPC-treated water: 1000ml H_2O with 1ml DEPC, microwaved for 1hr.

[6] 10x MOPS: pH 7.0, 4.1g Na Acetate (0.05M), 3.7g Na EDTA (0.01M), 41.8g MOPS (0.2M) in 1ml DEPC in 1000ml H_2O.

of the sample. The ratio was determined by adding 5-10 µl of RNA to 1ml DEPC-treated H_2O and the absorbance at 260 and 280nm was measured. The purity was assessed by the calculation; **A_{260} (RNA) - A_{260} (Blank)/ A_{280} (RNA).**

The value given was expressed as a ratio and a value of 1.5 ± 0.5 was assumed to be pure and free of DNA.

2.2.4.2 RT-PCR/PCR

Primers were assembled by Sigma-Genosys (London Road, Pampisford, Cambridgeshire, UK) and were constructed to correspond to a 300 bp region specific to HBD-2 RNA, as determined by the published sequence (NCBI, Entrez nucleotide query accession number Z71389).

Normal defensin primers

Anti-sense	AS HBD-2	-	GGAGCCCTTTCTGAATCGCA
Sense	S HBD-2	-	GCCATCAGCCATGAGGGT

MIMIC primers

S HBD-2 GCCATCAGCCATGAGGGT

Superprimer-

CCAGCCATCAGCCATTGAGGT(TTGAAACCAGCTGTGTGAAA)*

*(Junk region from MAGE2)

Cellular RNA was isolated from primary culture KC monolayers, BRT and LSE following the Qiagen RNAeasy isolation protocol as detailed above. 2µl of template RNA was subjected to RT-PCR using a Gibco BRL SS-one step reaction (Life Technologies, Paisley). In brief, 25 µl of 2x reaction buffer, 1µl of a 10µM primer mixture (HBD-23 and HBD-25), 1µl RT-Taq polymerase, 2µl template RNA and 21 µl RNAse-free H_2O was added together per reaction. cDNA was initially synthesised by incubating this reaction at 50°C for 30min in a Perkin-Elmer thermal cycler. cDNA was then denatured at 94°C for 2min and amplification was achieved by 35 cycles (determined the optimum number by analysis of reaction limits) of;

Denature: 94°C, 15s

Anneal: 55-60°C, 30s

Extension: 70°C, 1min.

Control PCR was carried out using a Qiagen PCR kit. In brief, 2μl of template RNA was added to 10μl of 10 x buffer, 20μl of 5 x Q buffer, 2μl of 10μM dNTP mixture, 1μl of 10μm HBD-23 primer, and 1μl of 10μM HBD-25 primer, 63.5μl H_2O and 0.5μl of template RNA. The amplification protocol was the same as used for RT-PCR as detailed above.

10μl of amplified samples were added to 5 μl of bromophenol blue loading buffer and run on a 1% agarose gel at 70 volts for 1hr. Samples were subsequently viewed using a UV transilluminator and checked against 100 bp markers (Life Technologies). Using the primers specific to defensin (HBD-23/25), revealed the need to quantitate expression as constitutive levels are expressed, hence a 'MIMIC' competitor sequence had to be constructed to add to the RT-PCR reaction to act as an internal comparison.

2.2.4.3 MIMIC PRODUCTION

For the production of a MIMIC fragment, RNA from defensin-positive samples was amplified using the standard defensin primer (HBD25) and a 'superprimer'. This primer was composed of the HBD-2-specific sequence, shown above, with an additional junk sequence derived from MAGE 6 melanoma antigen sequence (NCBI, Entrez nucleotide query accession number D32076). Hence the HBD-2 defensin primers 23 and 25 amplify a native region in the defensin-specific RNA while the superprimer and normal defensin primer amplify and produce a larger fragment from defensin-positive RNA. Further amplification of this competitor product revealed the necessity for cloning to obtain a pure sample of this fragment and to allow for the production of an RNA competitor.

2.2.4.4 LIGATION.

The excised MIMIC-specific band from an agarose gel was purified using the QIAquick spin gel extraction kit (Qiagen), resulting in a pure solution of MIMIC PCR product. This PCR product was ethanol-precipitated by adding 2 volumes of ice-cold ethanol (60μl of 100% research grade ethanol) to the purified PCR product and incubating at 70°C for 60min. The sample was then centrifuged for 15min at 5000xg and the supernatant removed. The pellet was washed once with 1ml 70% ethanol and re-spun for 5min at 5000xg. The pellet was then incubated at room temperature until completely dry when it was resuspended in $1/5^{th}$ the volume of the original PCR solution (6μl). Thereafter a PCR

Script Amp cloning kit (Stratagene, Gebouw California, Hogehilweg 15, Amsterdam, The Netherlands) was used to polish and ligate the PCR product into the plasmid vector (see Figure 8). Promoter sequence and restriction enzyme site information of cloning vector in Appendix 1.

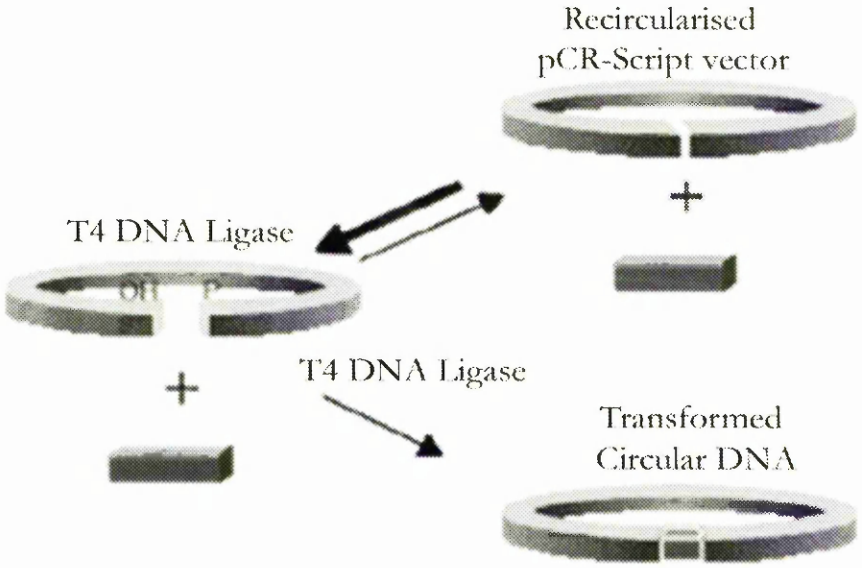

Figure 8: PCR-Script Amp phagemid cloning vector.

2.2.4.5 PREPARATION OF COMPETENT E. COLI

5ml of LB[7] broth was inoculated with a loop of *E. coli* XL10 cells (Life technologies, Paisley, UK) and incubated in a shaking incubator overnight at 37°C (200rpm). 100ml of LB broth was then inoculated with 1ml of this overnight culture and incubated with vigorous shaking (230rpm) at 37°C until the cell density equalled $5x10^7$ cells/ml (OD=0.5 measured at 210nm). Following centrifugation of this culture (4000xg at 4°C in a Sorval centrifuge) the cell pellet was resuspended in half the original volume, 50ml, of ice-cold 50mM $CaCl_2$/10mM Tris/HCl (pH 8.0) and incubated for 15min at 4°C. Cells were then centrifuged (4000xg for 5min) and resuspended in $1/15^{th}$ the original volume of $CaCl_2$Tris/HCl (pH 8.0) and stored on ice until use (12-24hr).

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[7] LB broth: 10g NaCl, 10g tryptone, 5g yeast extract in 1L deionised H_2O with pH7.0

2.2.4.6 *TRANSFORMATION*

The plasmid-containing insert was added to the competent E.coli (40ng of DNA per aliquot), and heat-shocked for 2min at 42°C. After adding 1ml of LB broth to each tube, this suspension was incubated at 37°C for 60min and inoculated onto Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), IPTG (isopropyl-1-thio-β-D-galactopyranoside) and Ampicillin-treated LB[8] agar plates and incubated overnight at 37°C.

Following selection of the colonies positive for insert (white), clones were picked off and spiked into 3ml LB/Amp broth (1/00 dilution of 1M Ampicillin) and incubated in a shaking incubator at 37°C (200rpm) for 24hr. The plasmid was extracted using the mini prep protocol (Qiagen) and ethanol precipitation of purified plasmid was carried out, as detailed above. Ethanol precipitation of the DNA achieved a five-fold concentration in the concentration of the plasmid DNA which was quantified using the A_{260} value.

2.2.4.7 *RESTRICTION ENZYME DIGEST*

A MAPSORT analysis of the defensin sequence in UNIX established that the Not-1 and Eco-R1 enzymes cut the plasmid adjacent to the insert (on opposite sides), but not the defensin-specific sequence. Therefore 0.2μl of Not-1, 0.2μl of EcoR1, 2μl of 10 x buffer (all obtained from Gibco, Life Technologies, Paisley), 7.6μl of H_2O and 10μl of purified plasmid (containing 2μg of DNA) was incubated for 1hr at 37°C. Ligation products were run on a 1% agarose gel, (70 volts for 1hr) to check size of insert and efficiency of ligation.

2.2.4.8 *TRANSCRIPTION*

Following the completion of ligation, 2μl of the linearised plasmid was added to 40μl T7 polymerase, 20μl of 2.5μM rNTPs[9], 10μl of 100μM DTT, 8μl H_2O and 20μl of 5 x

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[8]LB agar: 10g NaCl, 10g tryptone, 5g yeast extract, 20 g agar in 1L deionised H_2O with pH7.0 50μl of X-gal (0.25g of 1M X-gal dissolved in dimethyl farmamide) + 100μl of 1M IPTG was added per 100μg of medium.

[9] Ribonucleotide triphosphates, ATP (adenine), GTP (guanine), CTP (cytosine) and UTP (uracil). 10μl of each rNTP (100mM) in 360 μl of RNAse free H_20 gives a stock rNTP solution with 2.5μM of each.

transcription buffer (all obtained from Gibco Life Technologies, Paisley, Scotland, UK). This mixture was then incubated at 37°C for 90min. Following transcription, any remaining linear and circularised DNA was removed from the solution by the addition of DNAse, and DNA content was checked by PCR. The remaining solution was now composed of MIMIC-specific RNA, and could be used in a dilution series to determine the accuracy of the RT-PCR reaction both with and without the cellular RNA competitor.

2.2.4.9 COMPETITIVE RT-PCR

RT-PCR for HBD-2 /MIMIC competition was optimised by analysing the RT-PCR (SS one-step reaction, as detailed previously) at various time points, to determine the number of amplification cycles permitted before saturation of both products was achieved. The reaction details are as follows:

Reverse transcription: 30min 50°C, 94°C for 2min (1 cycle) and 32 cycles of

Denature: 94°C, 15s

Anneal: 55-60°C, 30s

Extension: 70°C, 1min

2.2.4.10 GEL ELECTROPHORESIS.

10μl of each reaction was loaded onto 4% agarose (1xTBE[10] buffer with1μg/ml ethidium bromide). Gels were run at 40 volts overnight (36hr) allowing separation and analysis of the defensin-specific and MIMIC-specific bands.

2.2.4.11 QUANTISCAN.

Densinometric analysis of the agarose gels was undertaken using Quantiscan software from Biosoft. In brief, Polaroid photographs of gels were scanned (at 400dpi's) using a flatbed scanner. The image was saved as a TIF file and imported into Quantiscan where densinometric analysis of the bands was carried out, each sample being analysed three times. These values were then used to assess a ratio of amplification of MIMIC (known) to cellular (unknown) in the RT-PCR reaction and this was then expressed in terms of molecules/μg of RNA added to the reaction. PCR amplification was also performed

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[10] 5x TBE buffer: 137g tris base, 68.75g boric acid, 50ml 0.5M EDTA (pH7.5) in 2.5L distilled H_20.

directly on all RNA samples without the inclusion of the reverse transcriptase step, to confirm that the observed gel product was derived from RNA.

2.2.5 STATISTICAL ANALYSIS/ SAMPLE DETAILS

For each experiment 1 donor foreskin was used to make a batch of KC monolayers or LSEs. One batch of BRT was also used for each experiment and a range of donors were used as detailed in table 8-16[11]. The RNA from 3 skin models (either KC monolayers, LSE or BRT) was isolated at each timepoint, for each test condition. The value expressed in each table entry and graph is the mean measurement of these three samples[12]. The between group differences (ie between control and organism treated samples) were measured using the students T-test, following application on the F-test to determine suitability of the standard deviations. The found value of T was expressed as a percentage probability (P) according to the appropriate tabulated values. A value of $P<0.01$ was determined to be significant and $P<0.001$ highly significant unless otherwise stated.

[11] KC donors 1-17 did not correspond to the same KCs donors used to make LSE batch 1-17.

[12] In the Figures the mean is expressed +/- the SEM.

2.3 RESULTS

2.3.1 VERIFICATION OF MIMIC SPECIFICITY

Initially a PCR competitor was created using the HBD-superprimer, with the same primer binding sites as HBD-2. However, as this would not give a directly quantitative value in RT-PCR, this DNA MIMIC (Figure 9) was inserted into a plasmid (Figure 10). Following reverse transcription and MAPSORT identification of those enzymes which would digest plasmid and not insert, a restriction enzyme digest of the plasmid showed that the plasmid RNA insert corresponds in expected size to the RT-PCR MIMIC product (Figure 11).

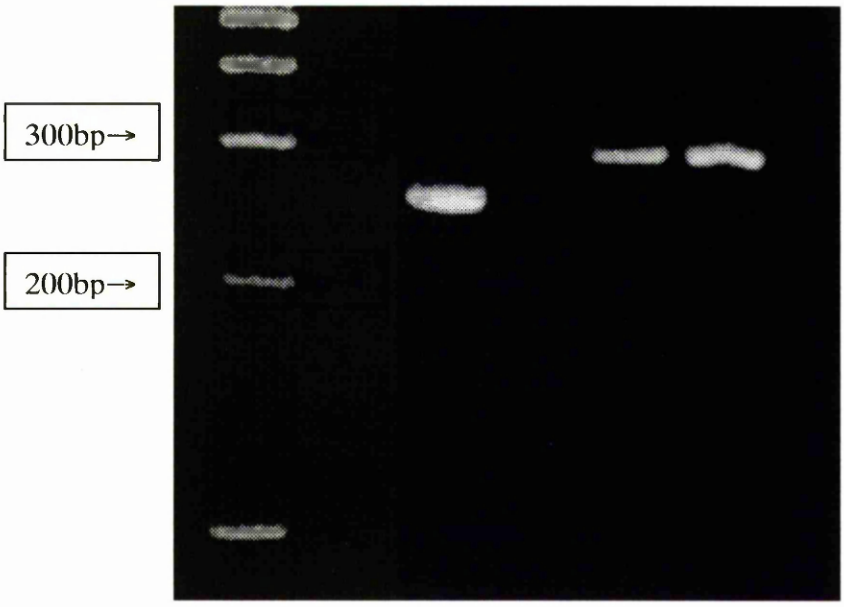


Figure 9: PCR compared to RT-PCR of MIMIC RNA.

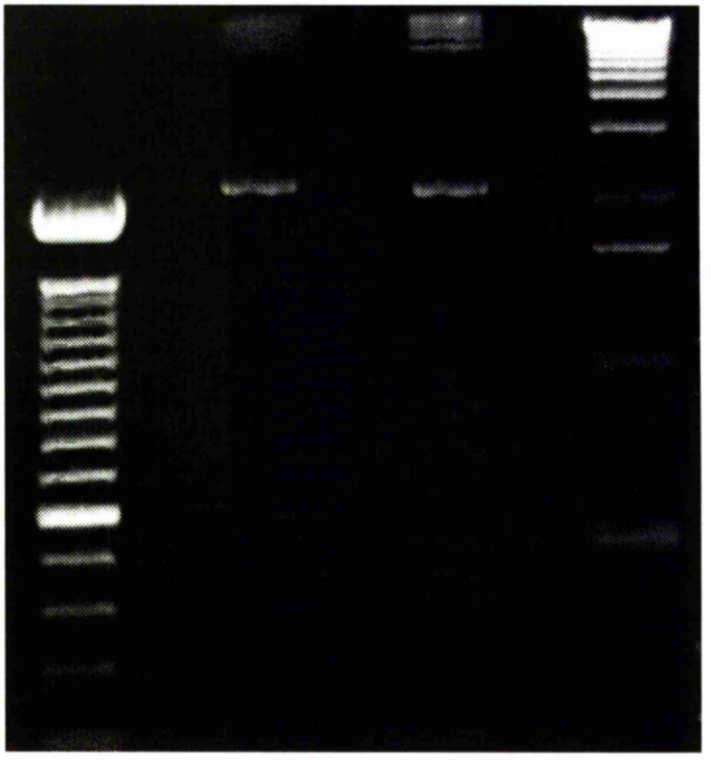
Lane 1: 100bp marker

Lane 2: RT-PCR of RNA using HBD-25 and HBD-23 primers

Lane 3: RT-PCR of RNA using superprimer and HBD-25 primer

Lane 4: PCR of Lane 3 DNA amplified with HBD-23 and HBD-25

Plasmid DNA MIMIC insert was converted to RNA by Not 1 digestion of the plasmid and T7 stimulated reverse transcription. Plasmid RNA was then isolated and DNAse treated. Purity of the RNA insert was determined by PCR of the RNA MIMIC produced, and a negative PCR reaction with the HBD-2 primers, which worked for RT-PCR, proved there was no DNA contamination.



Lane 1 Lane 2 Lane3 Lane4

Figure 10: Purified Plasmids containing MIMIC DNA insert.

Lane 1: 100bp ladder

Lane 2: Recircularised plasmid 1

Lane 2: Recircularised plasmid 2

Lane 4: 1Kbp marker

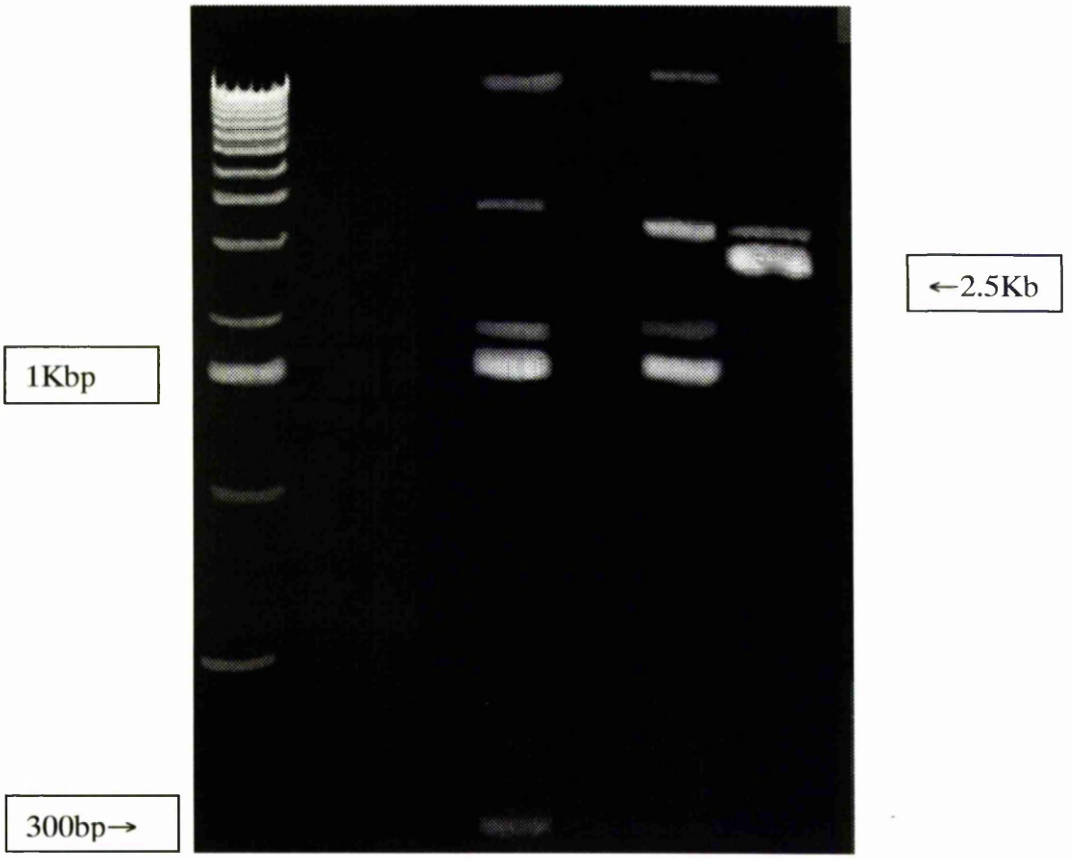


Figure 11: HBD-2 and MIMIC DNA products; comparison of weights.

Lane 1: 100Bp Ladder.

Lane 2: Not 1-digested plasmid reverse transcribed

Lane 3: Not 1-digested plasmid.

Lane 4: Undigested plasmid

The sequence of MIMIC, HBD-2 competitor was determined via T7 promoted automated sequencing of the MIMIC PCR product in the plasmid vector (Appendix 1). To check that the sequence of the competitor was as expected, the plasmid was linearised with Not 1 (on the opposite side of the insert) and sequenced with T7 polymerase. The ASHBD-2 and SHBD-2 primer binding sites remained intact and aside from the 'junk' inserted sequence, the MIMIC sequence (sequence 1) was identical to the HBD-2 published sequence (sequence 2) which the ASHBD-2 and SHBD-2 oligonucleotides amplify (Accession no AF071216).

The RNA MIMIC was quantified using the A_{260}/A_{280}. The initial stock and a 1/10 dilution of the stock was quantified by this technique as the sensitivity was not valid with an absorbance of less than 0.001. The MIMIC stock was calculated to contain 599.26μg/ml of RNA. Having verified the MIMIC sequence, it was possible to determine the molecular weight (Mwt) of 1 molecule of MIMIC by the sequence information available and the quantity of RNA available in the stock (Table 6 & Table 7). By converting thymine to uracil it was possible to determine that the Mwt of MIMIC was 56163 daltons; hence the stock solution contained 3.88037×10^{12} molecules/μl (See Calculation 1).

Table 6: Quantitation of MIMIC RNA by A_{260}/A_{280} ratio.

SAMPLE	A_{280} Blank	A_{280} RNA	A_{260} RNA	Ratio	Quantity (μg/ml)
STOCK	0.119	0.168	0.071	1.448	599.26[13]
1×10^{-1}	0.118	0.159	0.008	1.2796	35.352[14]

[13] A260 value x dilution factor (191) x 44.19 = RNAμg/ml

[14] A260 value x dilution factor (100) x 44.19 = RNA μg/ml

Sequence 1: MIMIC insert sequence

[15]**GGCGGC**CGCT CTAGCCC[16]**GCC** **ATCAGCCATG** **AGGGT**[17]**TTGAA**
ACCAGCTGTG **TGAAAGTCCT** **GCACCCTTGT** ATCTCCCTTC
TCGTTCCTCT TCATATTCCT GATGCCTCTT CCAGGTGTTT
TTGGTGGTAT AGGCGATCCT GTTACCTGCC TTAAGAGTGG
AGCCATATGT CATCCAGTCT TTTGCCCTAG AAGGTATAAA
CAAATTGGCA CCTGTGGTCT CCCTGGAACA AAATGCTGCA
AAAAGCCATG AGGAGGCCAA GAAGCTGATG [18]**TGGCTGATGC**
GGATTCAGAA **AGGGCTCCGG**

Sequence 2: HBD-2 amplicon sequence

[19]**GCC** **ATCAGCCATG** **AGGGTCTTGT** ATCTCCCTTC
TCGTTCCTCT TCATATTCCT GATGCCTCTT CCAGGTGTTT
TTGGTGGTAT AGGCGATCCT GTTACCTGCC TTAAGAGTGG
AGCCATATGT CATCCAGTCT TTTGCCCTAG AAGGTATAAA
CAAATTGGCA CCTGTGGTCT CCCTGGAACA AAATGCTGCA
AAAAGCCATG AGGAGGCCAA GAAGCTGATG [20]**TGGCTGATGC**
GGATTCAGAA **AGGGCTCC**

---

[15] Not 1 Binding site of plamid

[16] SHBD2 Primer binding site

[17] Junk mimic sequence

[18] ASHBD2 Primer binding site

[19] SHBD2 Primer binding site

[20] ASHBD2 binding site

Table 7: Number of individual bases in the MIMIC DNA sequence.

BASE	MOLECULAR WEIGHT	NO OF BASES IN INSERT	TOTAL Mwt (AMU)
ADENINE	135.14	68	9189.52
URACIL[21]	112	79	8848
GUANINE	151.13	71	10730.23
CYTOSINE	111.10	73	8110.3

Calculation 1: Determination of the number of MIMIC RNA molecules in the purified RNA stock.

Total Mwt of bases = 36878.05 atomic mass units (AMUs) for the 291 base product
Total Mwt for the sugar phosphate backbone of the RNA molecule = 291 x (PO_3 + Ribose; 78 + 115 =193) = 56163 AMUs

To calculate the number of molecules in the RNA stock the following equation was utilised.
[Concentration of stock (in g/L) x MW (in g)] x Avogadro's number = Molecules per litre

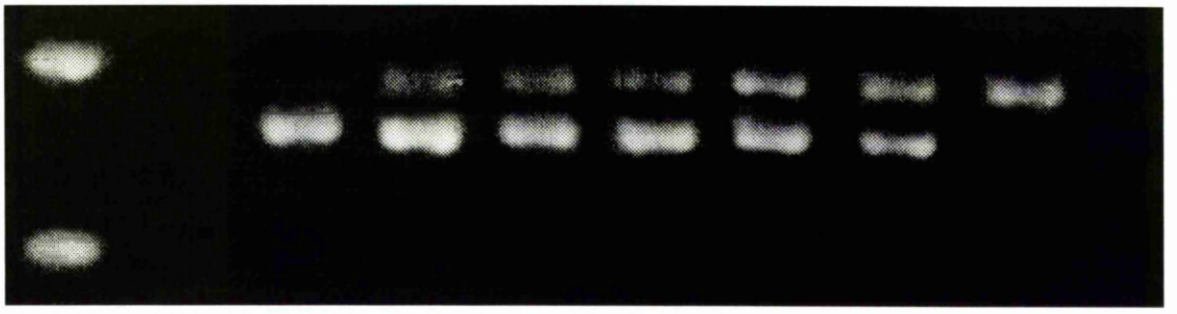
- Concentration of MIMIC stock = 599.26059µg/ml
- Total Mwt of RNA MIMIC = 93041.05 AMU's
- Avogadro's Number = 6.022 x 10^{23} therefore:

[599.26059/ 93041.05 (x1000)] x 6.022 x 10^{23}
= 3.877 x 10^{18} molecules/L
= 3.877 x10^{12} molecules/ µl

[21] Uracil replaces Thymine in the RNA sequence

By carrying out RT-PCR with a range of amplification cycles it was possible to determine the optimum RT-PCR reaction conditions, stopping the reaction before either the native HBD-2 mRNA or the MIMIC molecule reached its stationary phase of amplification. The optimum number of cycles was determined to be 32 as stated in the Methods (p67).

By densinometric analysis using Quantiscan it was possible to achieve a ratio for HBD-2 amplification against MIMIC amplification. From this ratio it was possible to establish how many molecules of HBD-2 were in the starting RNA sample which could then be standardised against the total RNA value. This was tested by diluting KC cellular RNA and carrying out RT-PCR with a standard, known dilution of MIMIC (Figure 12). As can be seen from Figure 13 the observed and expected results obtained are comparable, with a similar amplification relationship obtained even when diluting the cellular RNA.



Lane 1 Lane 2 Lane 3 Lane 4 Lane 5 Lane 6 Lane 7 Lane 8

Figure 12: RT-PCR of RNA dilution series with standard MIMIC added.

Lane 1: 100bp marker

Lane 2: KC RNA, no MIMIC RNA

Lane 3: 1/10 dilution of KC RNA + Standard MIMIC

Lane 4: 1/8 dilution KC RNA+ Standard MIMIC

Lane 5: 1/6 dilution KC RNA+ Standard MIMIC

Lane 6: 1/4 dilution KC RNA + Standard MIMIC

Lane 7: 1/2 dilution KC RNA+ Standard MIMIC

Lane 8: No dilution KC RNA + Standard MIMIC

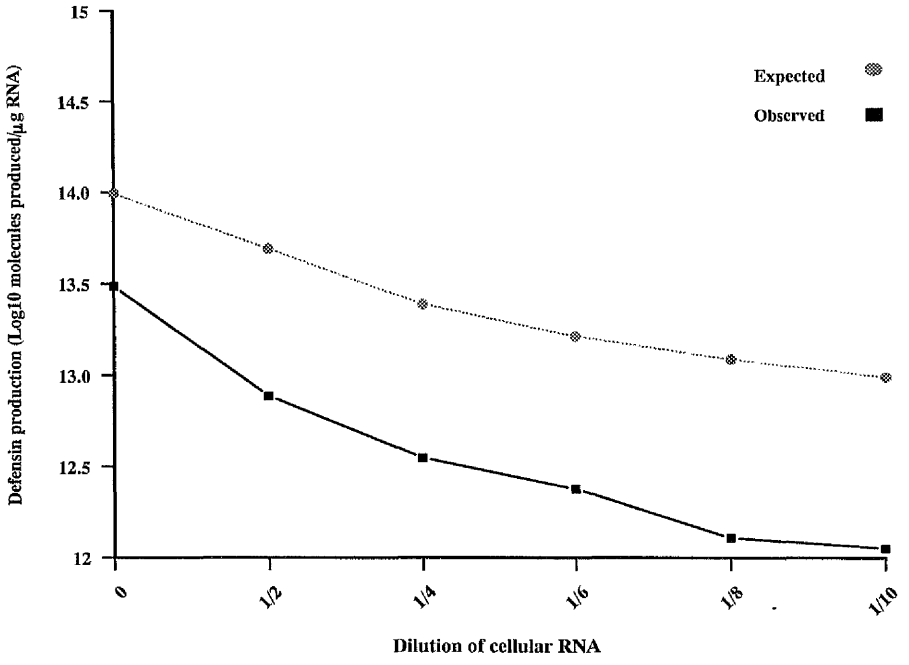


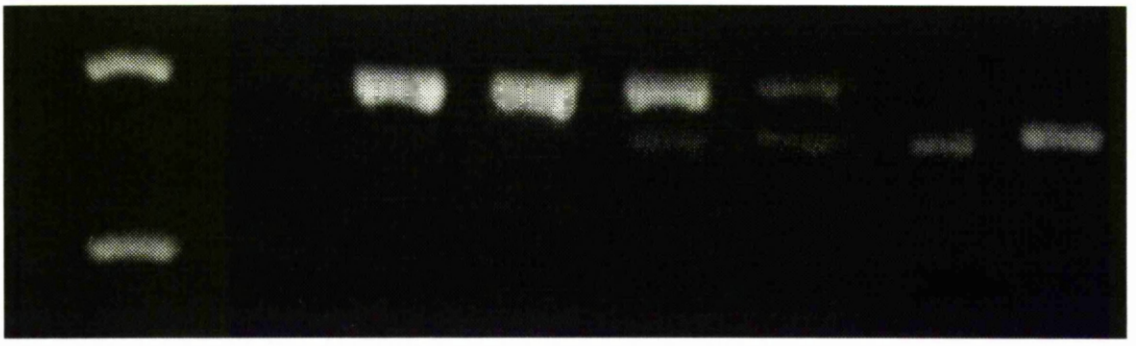
Figure 13: HBD-2 production by KC monolayers determined by competitive RT-PCR (Run 1).

To further reiterate the relationship of this RT-PCR reaction (Figure 13) a MIMIC dilution series with standard KC RNA was subjected to RT-PCR (Figure 14). As can be seen from Figure 15 there is some difference between the observed and expected results obtained. Although not overlying the expected results, the curve indicates a valid relationship between the dilution of the MIMIC and its ability to be measured by quiantiscan and RT-PCR. The loss of specificity of the MIMIC due to dilution did, however, indicate the sensitivity of this assay to dilution of MIMIC RNA; thus when carrying out quantitative RT-PCR a standard dilution of MIMC was included, with dilution of the target RNA occurring when necessary.

Having proven:

1. The purity of the MIMIC RNA stock,
2. The specificity of the ASHBD2 and SHBD2 primers for the MIMIC and cellular HBD-2 mRNA
3. The dynamics of the RT-PCR reaction and
4. The validity of the densinometric analysis

MIMIC stock was then used in RT-PCR reactions at a dilution determined by the target range of HBD-2 expression.



Lane 1 Lane 2 Lane 3 Lane4 Lane5 Lane6 Lane7

Figure 14: RT-PCR of MIMIC dilution series with standard KC RNA added.

Lane 1: 100bp marker

Lane 2: MIMIC RNA, no KC RNA

Lane 3: 1/2 dilution of MIMIC + Standard KC

Lane 4: 1/4 dilution of MIMIC + Standard KC

Lane 5: 1/6 dilution of MIMIC + Standard KC

Lane 6: 1/8 dilution of MIMIC + Standard KC

Lane 7: 1/10 dilution KC RNA+ Standard MIMIC

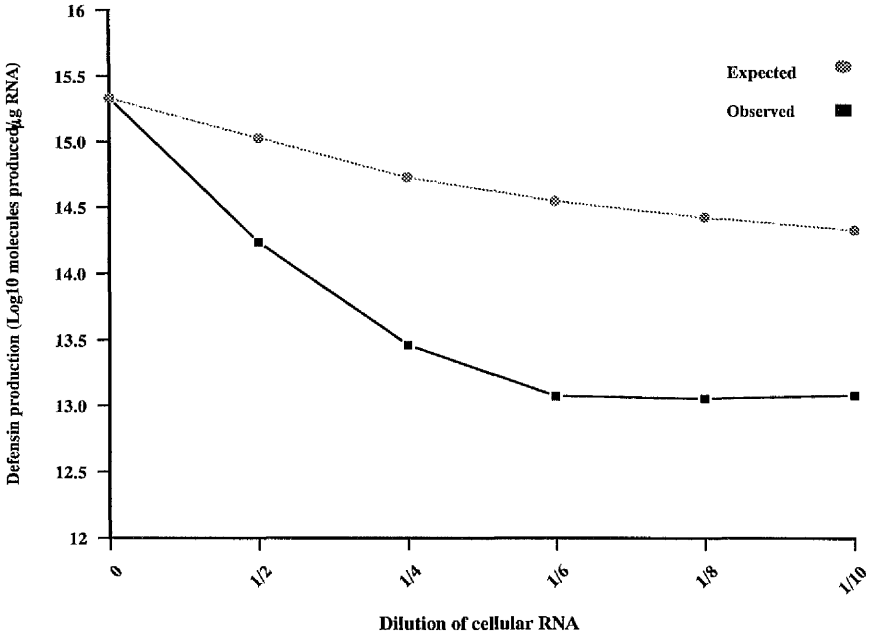


Figure 15: HBD-2 production by KC monolayers determined by competitive RT-PCR (Run 2).

2.3.2 CONSTITUTIVE PRODUCTION OF HBD-2 IN SKIN MODELS

The three skin models, LSE, BRT, and KC monolayers were assayed for constitutive production of HBD-2. MEM was added as a control to the dorsal surface of both the LSE and BRT and the normal MEM medium was considered a control for KC monolayers. The basal levels of HBD-2 were assayed at 24, 48 and 72hr in all these models and as can be seen from Table 8, 9 and 10 a wide range in HBD-2 expression was detected between different KC donors. The pattern of constitutive HBD-2 expression by KCs can be seen in Figure 16 and it should be noted that these experiments were initiated when the cell population was considered to be confluent, normally following 11-14 days incubation. Donor differences in KC HBD-2 expression were detected at 24hr where the range of production was 3.16×10^{9}-5.011×10^{14} molecules of HBD-2/μg KC RNA (Table 8)[22]. Hence constitutive expression of HBD-2 mRNA can be detected in KCs with a non-significant decrease in HBD-2 expression, compared to the 24hr level of expression, observed following 72hr incubation.

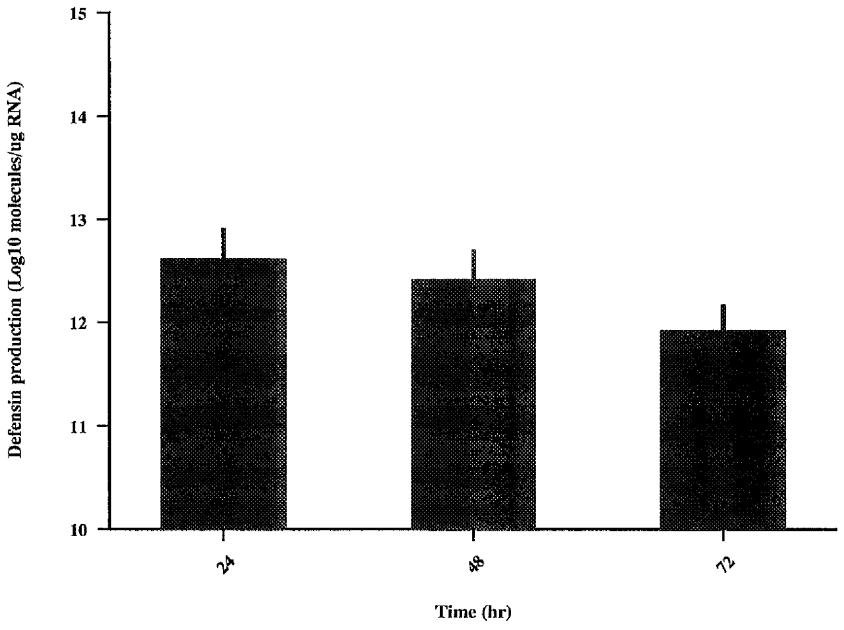


Figure 16: Constitutive HBD-2 expression on KC monolayers (Average of 10 donors).

[22] Each figure represents a single value of HBD-2 production for 1 KC monolayer.

Table 8: Range of constitutive HBD-2 expression by KC monolayers from 10 donors (Log10 Molecules/μg RNA).

	Time(hr)		
Donor	**24**	**48**	**72**
1	11.690	12.020	11.290
2	12.758	12.735	11.773
3	12.086	12.044	11.914
4	10.326	9.920	9.0698
5	13.321	12.814	12.611
6	15.229	14.561	13.663
7	13.963	14.021	12.215
8	13.320	12.814	12.611
9	10.326	9.9206	10.007
10	13.145	13.282	13.433

Table 9: Range of constitutive HBD-2 expression by LSEs from 6 donors (Log10 Molecules/μg RNA).

	Time (hr)		
Donor	**24**	**48**	**72**
1	12.812	14.014	13.159
2	11.795	13.221	13.326
3	10.557	12.829	13.265
4	13.168	14.014	13.159
5	12.739	13.077	12.759
6	11.920	13.398	11.753

The pattern of HBD-2 expression for BRT and LSE indicates a slight increase in expression at 48hr incubation, compared to 24hr, with a subsequent decline at 72hr for the LSE (Figure 17) and BRT (Figure 18). However as can be seen by comparing table 9 and 10 with figure 17 and 18, this pattern is not consistently observed and was not statistically significant. The LSE at the '24hr incubation' time point has been at the air/ liquid interface for in excess of 6 days (most commonly 10 days) prior to commencement of the experiment[23]. Hence the differences in KC viability and growth may affect the production of HBD-2. The BRT '24hr incubation' time point is 24hr post-excision, or at most 24hr post-day of excision, as all tissue were collected and processed on the day of excision. Both the BRT and LSE exhibit donor differences in basal expression of HBD-2 with the LSEs ranging from 1.58 x 10^7-1.26 x 10^{15} molecules/μg RNA (Table 9) and BRT ranging from 7.94 x10^{10}- 3.98 x 10^{14} molecules/μg RNA (Table 10). Comparison of the models shows that LSE produces slightly more HBD-2 than both KCs and LSEs (Figure 19).

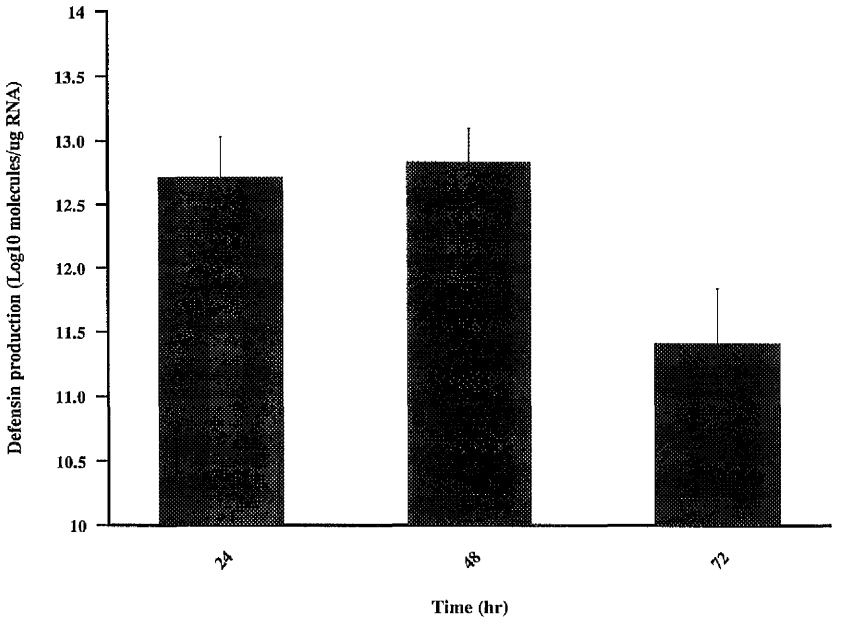


Figure 17: Constitutive HBD-2 expression on LSE (average of 6 donors).

[23] A KC monolayer of each of the KC seeded on the LSE was used to approximate the growth of the KCs on the LSE.

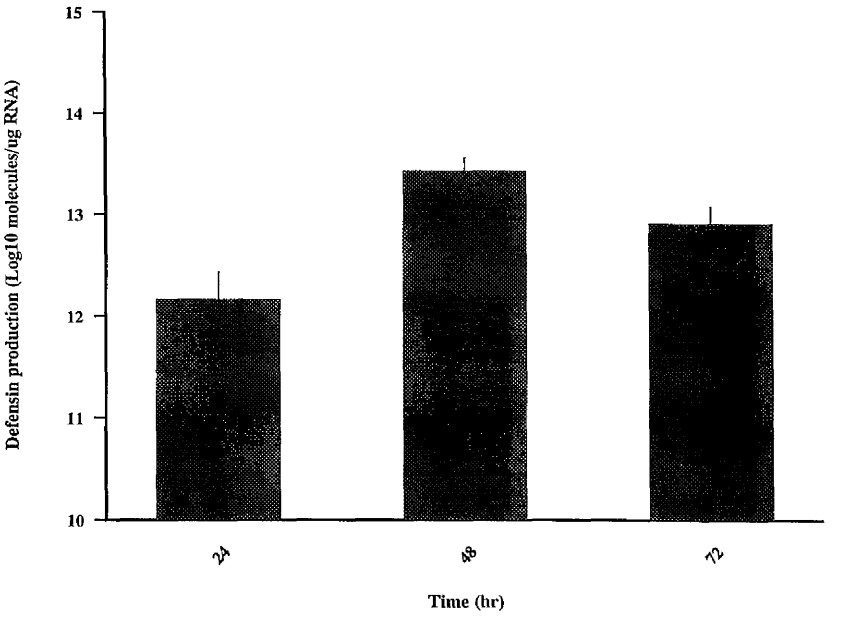


Figure 18: Constitutive HBD-2 expression on BRT (average of 6 donors).

Table 10: Range of constitutive HBD-2 expression by BRT from 6 donors (Log10 Molecules/µg RNA).

	Time (hr)		
Donor	**24**	**48**	**72**
1	12.812	14.014	13.159
2	11.795	13.221	13.326
3	10.557	12.829	13.265
4	13.168	14.014	13.159
5	12.739	13.070	12.750
6	11.820	13.398	11.750

As can be seen from Figure 19 there was slight differences in the overall patterns of expression of HBD-2 in the three skin models. The only notable point is that HBD-2 expression on BRT is lower at 24hr than all the other skin models at the same time and that the LSE and KC monolayers demonstrate a drop in HBD-2 production following 72hr incubation, compared to levels detected at 24hr.

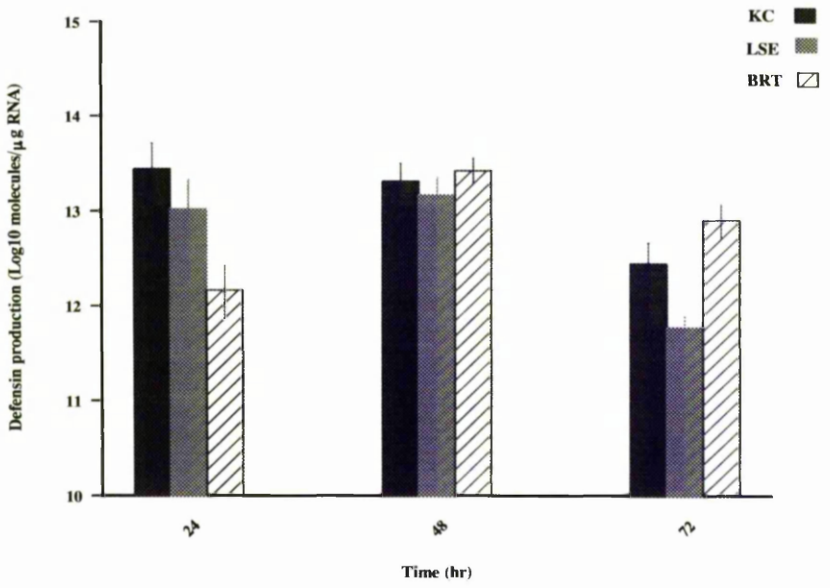


Figure 19: Constitutive HBD–2 expression on KC, LSE and BRT.

2.3.3 HBD-2 EXPRESSION IN KC MONOLAYERS

To establish the inducibility of the KC monolayers to inflammatory mediators, the cells were washed and tested by the exogenous addition of TNFα (10μg added per ml of MEM). Donor 11 KCs show increased, significant (P<0.001) upregulation of HBD-2 expression in response to TNFα (Figure 20) and this magnitude of response increased with time. However donor differences in reaction to TNFα did exist and using donor 12 KCs TNFα did not stimulate HBD-2 production until 72hr incubation. (Figure 21). This difference in KC reaction to TNFα reiterated the fact that there appeared to be donor specific HBD-2 production and this was observed in response to the addition of exogenous compounds. However as TNFα was only tested on 2 KC donors it is difficult to judge whether this difference was entirely donor specific.

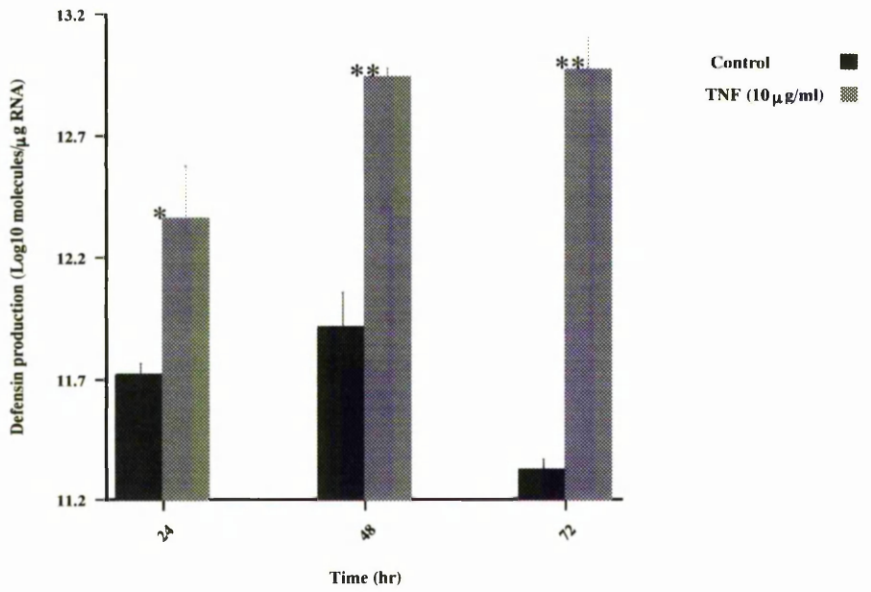


Figure 20: HBD-2 production on KC monolayers, donor 11, in response to TNFα.

* Denotes significant difference compared to control (P<0.01)

**Denotes highly significant difference compared to control (P<0.001)

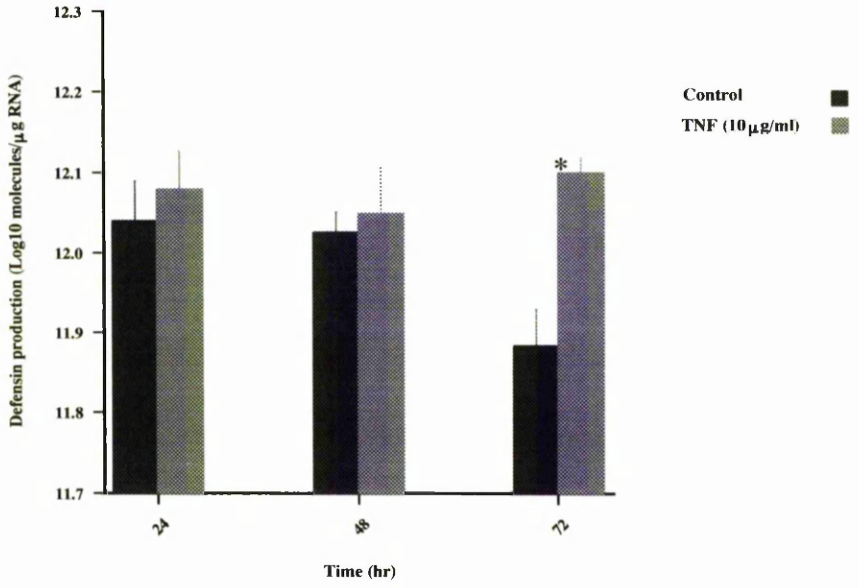


Figure 21: HBD-2 production on KC monolayers, donor 12, in response to TNFα.

* Significant difference to control (P<0.01)

Table 11: HBD-2 expression in reponse to 10µg/ml of *M. furfur* cell wall (Log 10 molecules/µg RNA). Range of expression between 3 donors.

	Time (hr)					
Donor	**24**		**48**		**72**	
	Control	**cell wall**	**Control**	**cell wall**	**Control**	**cell wall**
13	13.098	12.695	13.239	12.991	13.121	12.796
14	13.531	13.278	13.152	12.922	13.182	12.711
15	13.044	12.944	13.115	12.199	13.161	13.165

To investigate the possibility that *M. furfur* may interact with KCs to alter HBD-2 expression *M. furfur* and *M. furfur* components were added to KC monolayers to determine if the organism altered the expression of HBD-2. Firstly donor 13 KCs were co-incubated with two dilutions of *M. furfur* cell wall suspension (100μg/ml + 10μg/ml in MEM). These KCs demonstrated a dose-dependent decrease in HBD-2 expression in response to the cell wall (Figure 22). This inhibition was achieved at 24hr with 10μg/ml on donor 14 KCs (Figure 23) and this was found to be borderline significant ($P > 0.01 < 0.05$). Comparison of these two figures (Figure 22 & Figure 23) indicate the difference in magnitude of expression between donors in response to *M. furfur* cell wall and demonstrate that this reaction was donor specific. In addition it should be noted as as can be seen in table 11, that this suppression while significant at 72hr for donors 12 & 13 was not consistently observed. Hence putative suppression of HBD-2 expression by *M. furfur* cell wall was in direct contrast to the effect seen in the response of donor 15's KCs to *C. albicans* cell wall. This donor initiated an increase in HBD-2 transcription following 48 and 72hr incubation (Figure 24) with *C. albicans* cell wall (10μg/ml). However again as this was only tested on 1 batch of KC monolayers this effect cannot be verified.

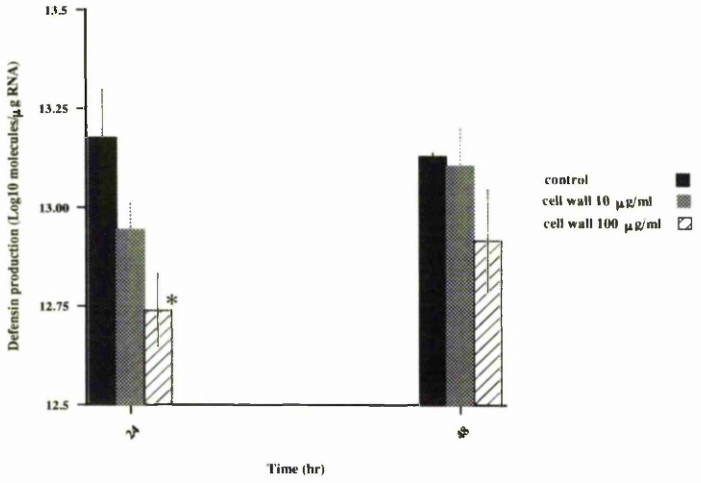


Figure 22: HBD-2 production on KC monolayers, donor 13, in response to *M. furfur* cell wall (0-100µg/ml).

* Borderline significantly different to control (P>0.01<0.05) [24]

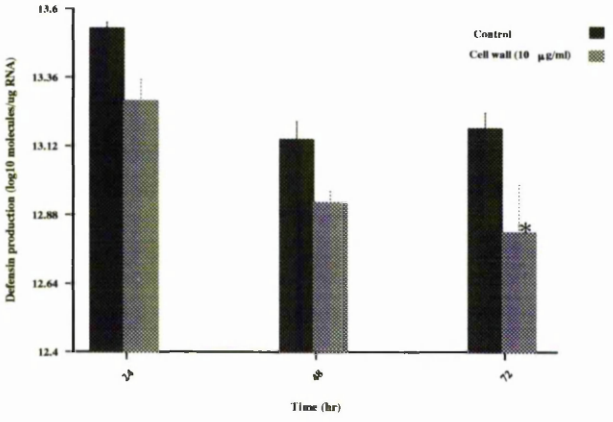


Figure 23: HBD-2 production on KC monolayers, donor 14, in response to *M. furfur* cell wall (10µg/ml).

* Borderline significantly different to control (P>0.01<0.05)

[24] Found value of T was so close to the tabulated 'signifcant' value, with appropriate degrees of freedom, as to warrant comment.

To compare differences in cell wall and whole cells, viable *M. furfur* (10^4cells/ml MEM) were added to the dorsal surface of KCs. In donor 16 *M. furfur* initiated a decrease in HBD-2 expression at 24hr followed by an increase (Figure 25). The differences in HBD-2 expression between donors is best seen in Table 12 and although not always absolute there did appear to be a trend for *M. furfur* to increase HBD-2 transcription following 48hr incubation. This difference was not however measured as statistically significant. Out of the 5 KC donors tested, 3 demonstrated this pattern of slight inhibition with subsequent stimulation. Therefore bearing in mind the lack of statistical significance in the results all that could be noted is that a trend did exist which was subject to donor variation. The capacity of viable *M. furfur* to suppress and stimulate defensin production was examined further by the application of whole *M. furfur* cells, a killed whole cell suspension (10^4cells/ml MEM), to KC monolayers[25]. These cells initiated inhibition of HBD-2 expression by the KCs on donor 21 (Figure 26). However as this was only one donor no conclusions can be drawn from the data.

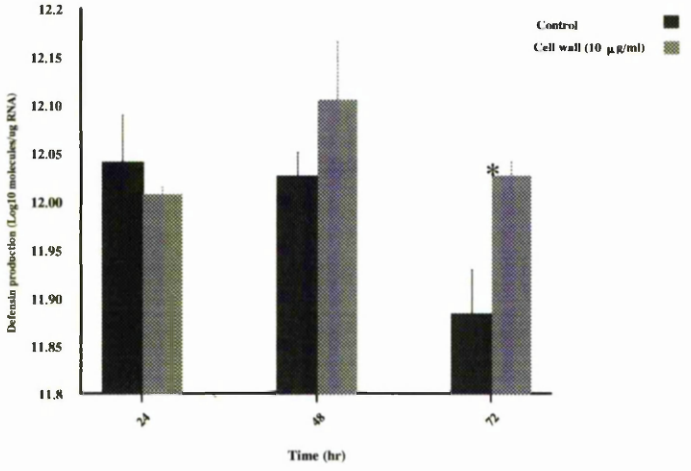


Figure 24: HBD-2 production on KC monolayers, donor 15, in response to *C. albicans* cell wall.

* Significant difference from control ($P<0.01$)

[25] Unless otherwise stated this cell number of killed whole cells was always used.

Table 12: HBD-2 expression by KC monolayers over time in response to viable *M. furfur* (Log 10 molecules/μg RNA). Range of expression between 5 donors.

Donor	Time (hr)					
	24		48		72	
	Control	*M.furfur*	Control	*M. furfur*	Control	*M.furfur*
16	15.241	14.789	14.687	14.822	16.654	13.935
17	12.045	12.067	12.026	12.104	11.883	12.026
18	12.952	12.737	13.025	13.218	13.359	13.477
19	13.193	13.037	13.460	13.483	12.414	13.411
20	13.104	13.258	12.613	14.119	13.0449	13.316

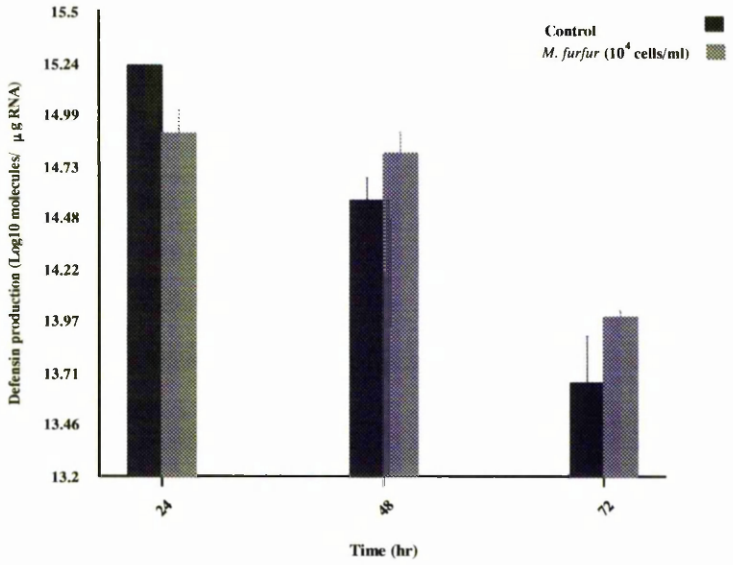


Figure 25: HBD-2 production on KC monolayers, donor 16, in response to viable *M. furfur* .

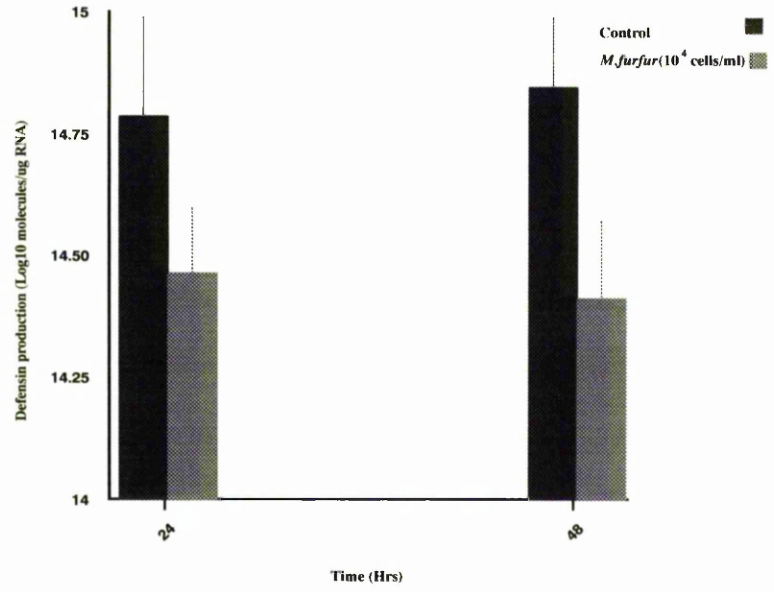


Figure 26: HBD-2 production on KC monolayers, donor 21, in response to killed *M. furfur*.

2.3.4 HBD-2 EXPRESSION ON LSE

To establish whether HBD-2 expression could be induced on the LSE, three dilutions of TNFα (15 μl of 1, 10 or 100 μg/ml of TNFα diluted in MEM) were added to the dorsal surface of LSE (donor 7). With this LSE, TNFα induced HBD-2 expression at 24hr. However statistical analysis could not be performed on these results due to lack of sample size (Figure 27)[26], although it did appear that in this donor, in the first 24hr, TNFα did increase HBD-2 production. To test the effect of fungal growth on the LSE, viable *M. furfur* was added to the dorsal surface of the LSE and the RNA analysed. There was a depression of HBD-2 production in response to the organism at 24hr on almost all donors, with a subsequent slight increase in defensin transcription (Figure 28) similar to the response seen with some KC monolayers. However as can be seen by comparing other donor values (Table 13) and in common with the KC response to viable *M. furfur*, no statistical significance was observed in these results. However all of the donors tested did exhibit a slight decrease in HBD-2 production at 24hr with a relative increase at 72hr (compared against time and donor matched controls). As these alterations were not significant at any time, and as with the KC data, these measurements merely indicated a trend in the HBD-2 production of the LSE in response to *M. furfur*.

[26] Only tested on one batch of LSE's from one donor. Duplicate samples taken at each timepoint for each test condition.

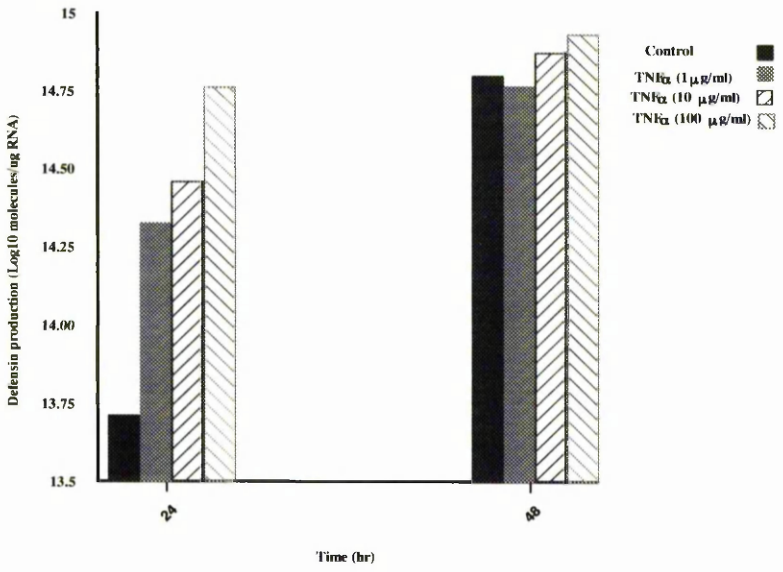


Figure 27: HBD-2 production on LSE, donor 7, in response to TNFα.

To examine this trend further cell wall and killed whole cells were added to the LSE surface and HBD-2 expression was measured. *M. furfur* killed whole cells initiated a dose-dependent suppression of HBD-2 production in donor 14 (Figure 29) whereas *M. furfur* cell wall (10μg/ml) did not affect HBD-2 production in donor 15 (Figure 30). These results again were not statistically significant and merely indicate an individual LSE batch reaction to *M. furfur* components.

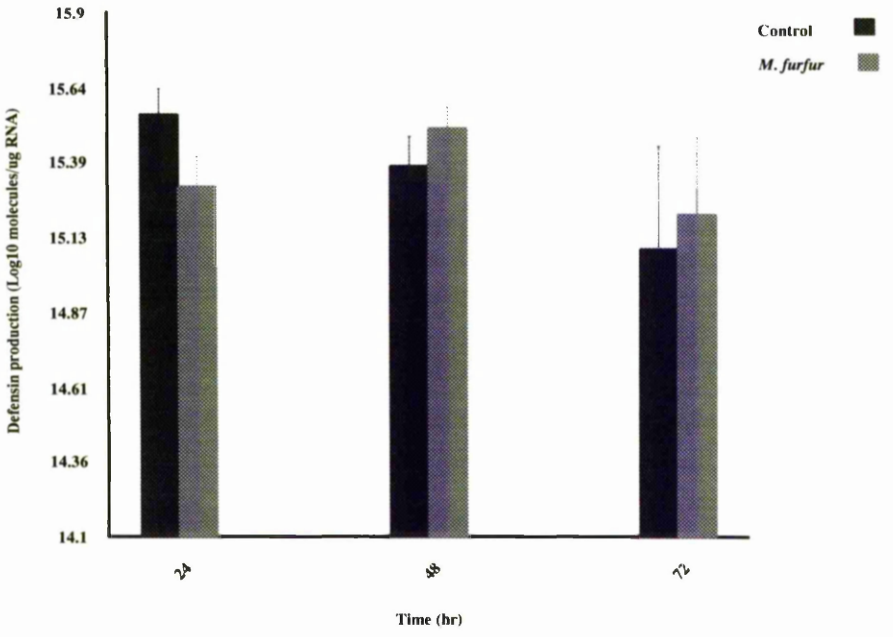


Figure 28: HBD-2 production on LSE, donor 8, in response to viable *M. furfur*.

Table 13: HBD-2 expression on LSEs in reponse to viable *M. furfur* (Log 10 molecules/μg RNA). Range of expression between 6 donors.

	Time(hr)					
Donor	**24**		**48**		**72**	
	Control	*M.furfur*	Control	*M.furfur*	Control	*M.furfur*
8	15.511	15.289	15.328	15.587	14.988	15.244
9	13.449	13.168	14.014	13.786	13.159	13.321
10	12.9153	12.569	12.439	12.722	12.399	12.895
11	14.023	13.843	13.648	14.022	13.583	13.769
12	13.779	13.491	13.661	13.657	13.753	13.761
13	12.973	12.630	12.488	13.145	[27]	

[27] No 72 hr data available for this donor due to loss of RNA.

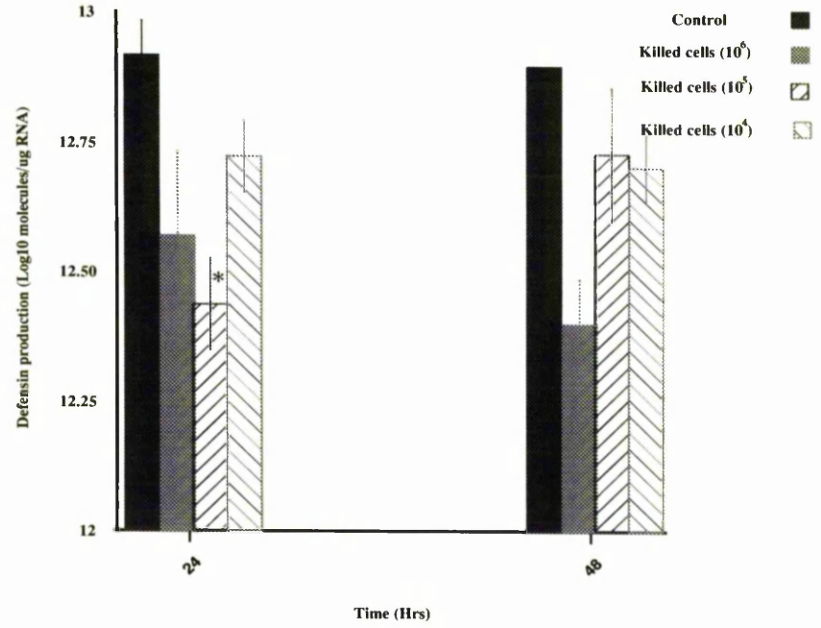


Figure 29: HBD-2 production on LSE, donor 14, in response to killed *M. furfur*.

*Signifact difference from control ($p<0.01$)

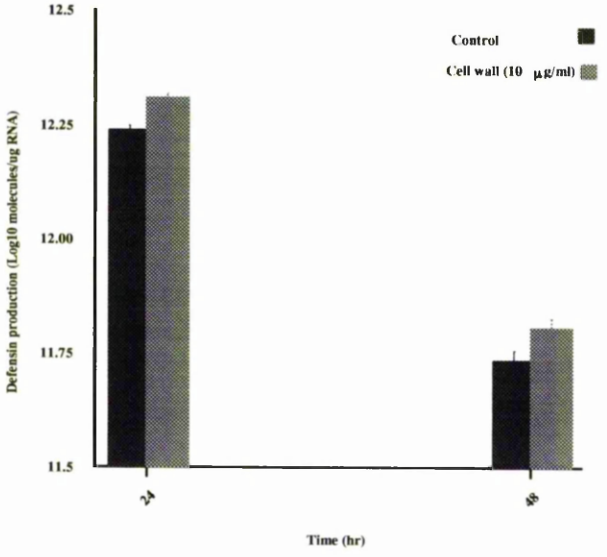


Figure 30: HBD-2 production on LSE, donor 15, in response to *M. furfur* cell wall.

To determine whether the LSE would respond to yeast cells, *C. albicans* was added to the dorsal surface of the skin model. The addition of *C. albicans* stimulated defensin production by the LSE following 48hr incubation in donor 16 (Figure 31), Figure 31 demonstrates that the ability of *C. albicans* to stimulate HBD-2 expression by the LSE is significant in donor 31 at 48hr. As can be seen from Table 14 this increased expression in response to *C. albicans* growth was identified on 2 LSE batches. However only one showed statistical significance- donor 16.

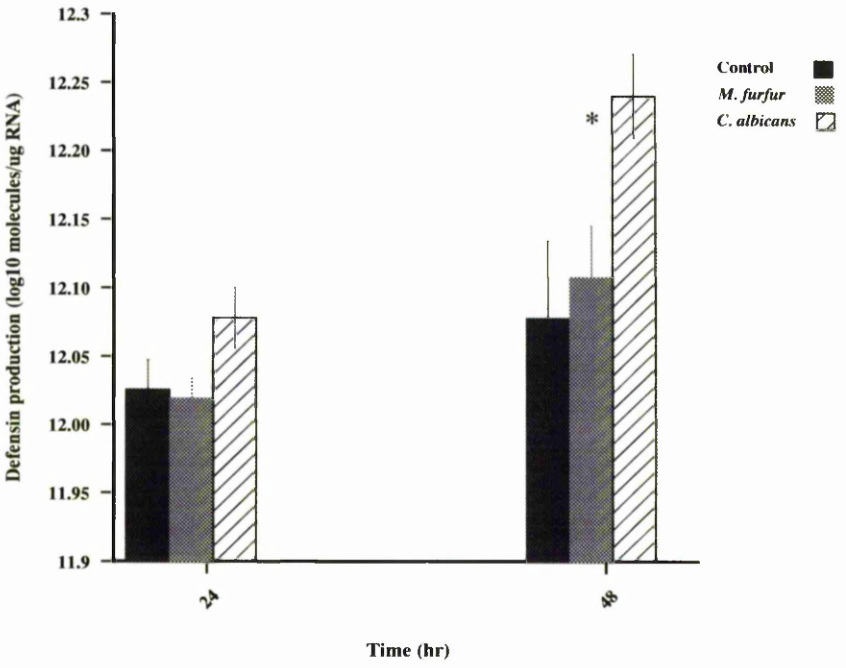


Figure 31: HBD-2 production by LSE, donor 16, in response to viable *M. furfur* and *C. albicans* growth.

* Significant difference from control ($P<0.01$)

Table 15: HBD-2 expression on the LSE in reponse to viable *M. furfur* and viable *C. albicans* (Log 10 molecules/µg RNA). Range of expression between 2 donors.

	Time (hr)								
	24			48			72		
Donor	Control	*M.furfur*	*C.albicans*	Control	*M. furfur*	*C. albicans*	Control	*M.furfur*	*C.albicans*
16	12.025	12.018	13.078	12.756	12.106	13.239	[28]		
17	10.186	9.061	11.923	15.589	16.463	17.874	15.883	16.704	17.549

[28] No 72hr data for this donor due to loss of RNA.

2.3.5 HBD-2 EXPRESSION ON BRT

Inducibility of HBD-2 expression by BRT was examined by adding 15µl of a 10µg/ml TNFα solution to the dorsal surface of the tissue no more than 24hr after excision. An increase in HBD-2 transcription was observed at 72hr, allthough the magnitude of the increase was less than that observed with the LSE and KC monolayers and was only statistically significant at 72hr (Figure 32).

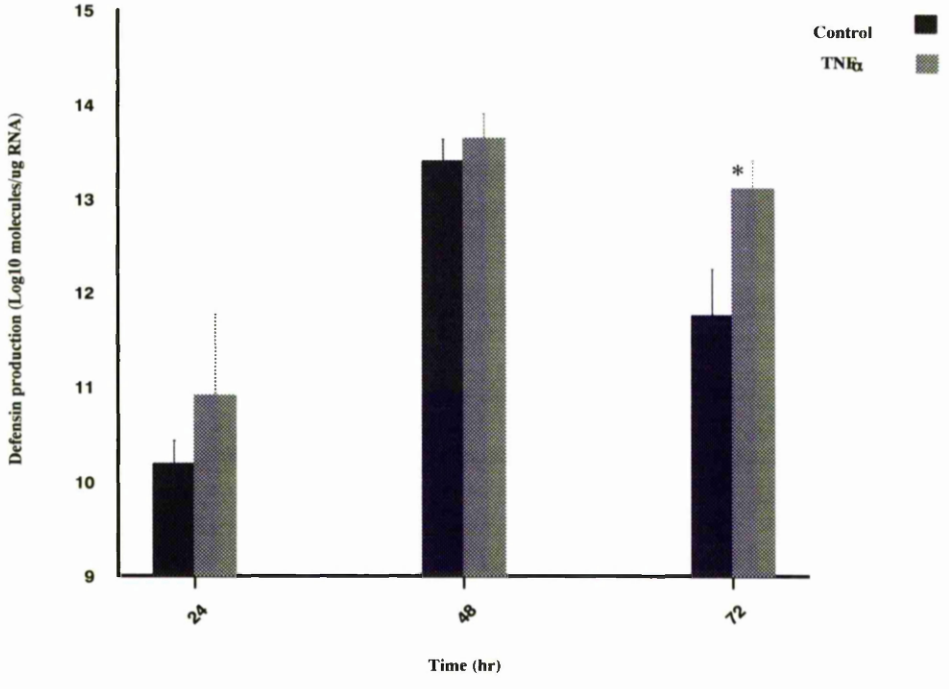


Figure 32: HBD-2 production on BRT, donor 7, in response to TNFα.

* Significant difference from control (P<0.01)

Growth of *M. furfur* on the dorsal surface of BRT resulted in a pattern of HBD-2 expression similar to that on LSE, with suppression of defensin expression at 24hr (Figure 33). However as can be seen from Table 16 this effect is not seen in all BRT donors and the effect was not consistent as suppression of HBD-2 expression was deteceted in donor 8 at 48hr. As with the LSE and KC data this effect was neither consistent or statistically significant. The BRT, as with the LSE, increased HBD-2 transcription at 24hr in response to *C. albicans* growth (Figure 34). This effect was generally observed in all donor BRT tissue tested (Table 17). Killed whole *M. furfur* and *C. albicans* cells did not greatly affect HBD-2 production on BRT donor 11 (Figure 35).

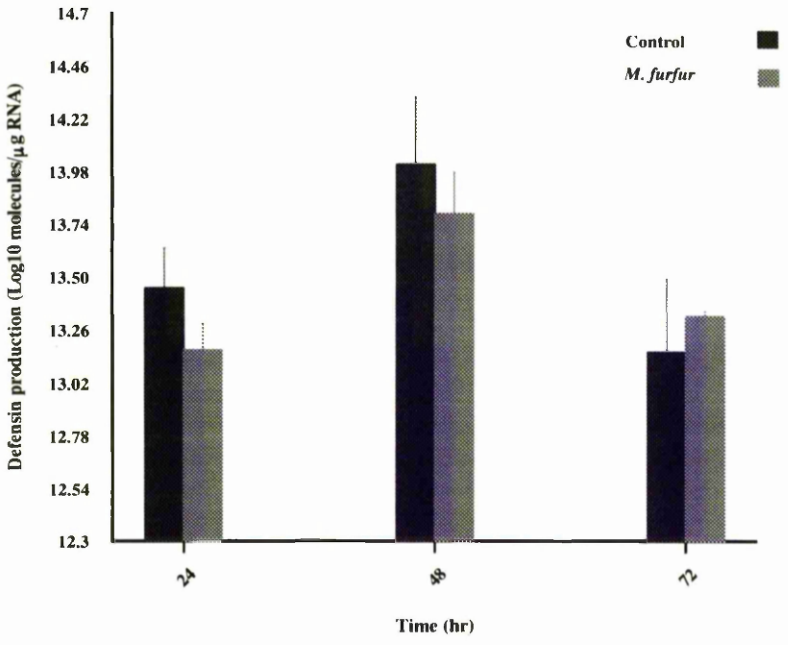


Figure 33: HBD-2 production on BRT, donor 8, in response to *M. furfur* colonization.

Table 16: HBD-2 expression on BRT in reponse to viable *M. furfur* (Log 10 molecules/µg RNA). Range of expression between 6 donors.

Donor	Time (hr)					
	24		48		72	
	Control	*M. furfur*	Control	*M. furfur*	Control	*M. furfur*
8	13.449	13.168	14.014	13.786	13.159	13.321
9	12.691	12.909	12.099	12.265	13.183	13.274
10	11.307	11.566	11.966	12.619	11.044	10.802
11	12.821	12.4524	13.102	13.172	12.907	13.124
12	13.576	12.159	13.326	13.076		
13	9.939	10.356	9.817	9.744		

Table 17: HBD-2 expression on BRT in reponse to viable *M. furfur* and *C. albicans* (Log 10 molecules/µg RNA). Range of expression between 3 donors.

Donor	Time (hr)								
	24			48			72		
	Control	*M.furfur*	*C.albicans*	Control	*M. furfur*	*C. albicans*	Control	*M.furfur*	*C.albicans*
14	10.189	9.974	11.981	12.595	13.512	13.672	11.811	13.212	13.914
15	12.739	12.254	13.057	13.079	13.013	13.127	13.048	12.810	13.146
16	13.198	13.385	13.555	14.014	13.829	13.053	12.639	13.159	13.363

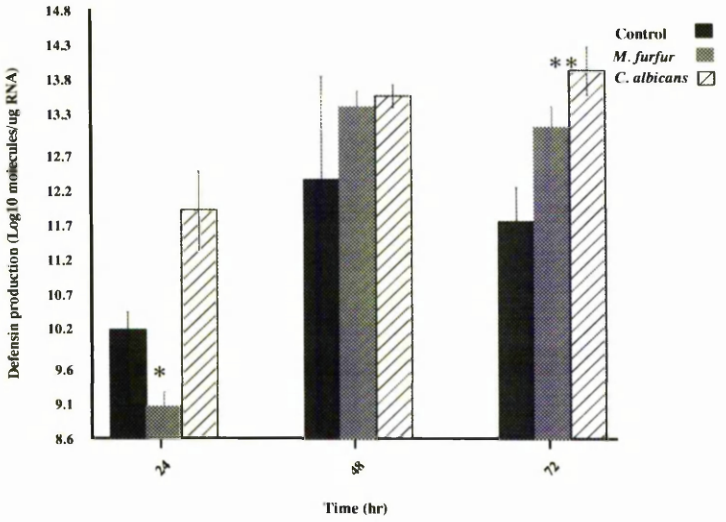


Figure 34: HBD-2 production on BRT, donor 14, in response to *M. furfur and C. albicans* colonisation.

* Significant difference from control (P<0.01)

** Highly significant difference from control (P<0.001)

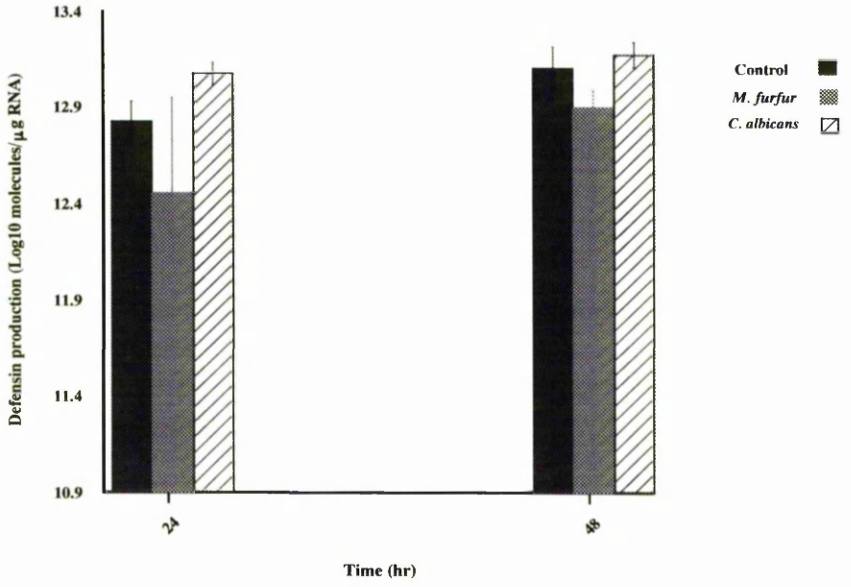


Figure 35: HBD-2 production on BRT, donor 17, in response to killed *M. furfur* and *C. albicans*.

2.4 DISCUSSION

The method developed here to assay HBD-2 transcription in the skin models has several advantages over other methods of quantitative RT-PCR analysis. The HBD-2-specific RNA competitor 'MIMIC' has two main advantages over semi-quantitative analysis using internal cellular standards, such as GAPDH (Bargon, 2000). Firstly, MIMIC was amplified with the same primers as the target. This negates the need to calculate any differences in primer binding efficiency, which would exist between, say GAPDH and HBD-2-specific primers. In addition, these cellular targets (GAPDH, actin, etc) change expression between cell populations making it impossible to obtain a comparative value for the measure of expression between cell populations. Only within group changes, those within the same population of cells can be assessed using internal competitors. By adding an external competitor it was possible to compare expression between groups of cells, and hence different models, as well as within groups. The experiments undertaken therefore had two main objectives: to establish the constitutive expression of HBD-2 in the three skin models, and to determine the alteration of HBD-2 expression in response to *M. furfur* and *M. furfur* cell walls.

Antimicrobial peptide expression has been demonstrated in normal skin where HBD-1 mRNA was detected *in situ*, and was shown to be located in suprabasal KCs (Fulton et al., 1997). HBD-2 expression has also been detected in punch biopsy samples of normal tissue, using anti-HBD-2 polyclonal antibody in immunohistochemistry (Ali, 2000). Although no peptide has been directly isolated from normal skin as is the case with HBD-1 (Schroder and Harder, 1999), it would seem that samples like gingival epithelia (Krisanaprakornkit et al., 2000), ocular epithelia (Lehmann et al., 2000) and skin epithelia constitutively produce both HBD-1 and HBD-2. This hypothesis is supported by the analysis of freshly isolated BRT epidermal mRNA which showed, using competitive RT-PCR with MIMIC, that HBD-2 is expressed at a constitutive level[29] and that this basal level of expression differs greatly between donors. This corresponds with the data relating to HBD-2 expression in lung epithelia, where donor differences in the intensity of HBD-2 anti-sense probe binding

[29] Results not shown:RT PCR was performed on freshly isolated RNA from 2 BRT donors. HBD-2 expression was not quantified however crude expression was detected.

were evident (Singh et al., 1998).

These results indicate that BRT expression of HBD-2 is slightly increased following 24hr incubation which may be due to inflammatory mediators that are produced in response to the trauma of excision. Excision, like wounding, will initiate the release a myriad of inflammatory mediators, some of which will induce IκBα activation and result in loss of NFκB inhibition. This will in turn initiate transcription of NFκB-dependent genes, one of which is thought to be that coding HBD-2, and therefore the physical act of excision may be responsible for the observed rise in HBD-2 expression following incubation of the tissue. The inflammation related to trauma may also be responsible for the basal HBD-2 expression, within 12hr of excision, as KCs *in vitro* have been shown to respond to TNFα with increased HBD-2 expression within 2hr of exposure (Harder et al., 1997). The paradox of whether HBD-2 is constitutively expressed in skin is not easily solved. By snap freezing punch biopsy material the mRNA present could be amplified and hence measured; however, the speed at which cells could be induced to produce HBD-2 would have to be accounted for.

KC monolayers, BRT and LSE all demonstrate constitutive defensin mRNA expression, following 24hr incubation. With regard to KCs, the 'constitutive' expression of HBD-2 may be due to culture conditions, with Ca^{2+} in the medium stimulating both differentiation and growth of the KCs. Culture conditions have been shown to stimulate defensin expression since medium from freshly isolated KCs does not contain detectable HBD-2 peptide, but following culture, peptide can be detected in the culture medium (J.M. Schroder, personal communication). KCs do give positive RT-PCR for HBD-2 expression directly following excision but if functional peptide cannot be detected perhaps the message is not being effectively translated, or at least is not being translated into physiologically detectable values. Experiments with lung epithelia cell lines compared with *in situ* expression experiments (Singh et al., 1998) demonstrate that *in vitro* mRNA expression and protein expression cannot always be extrapolated back to *in vivo* models. Thus HBD-2 expression, in common with HBD-1, appears to be constitutively expressed in skin as it is in various other tissues (Bals et al., 1998).

Until this research, no measure of the alteration of constitutive HBD-2 expression in skin

models had been undertaken. It is highly likely that the increases in BRT transcription of HBD-2 with time is due to post-surgery trauma of the KCs of the BRT. It has, however, been shown that HBD-2 expression is decreased in the area surrounding burn wounds (Milner and Ortega, 1999), a finding which does not correlate with the idea of a role for of HBD-2 as an essential factor in infection regulation. This down regulation of HBD-2 production may be due to a loss of KCs from the tissue surrounding the burn wound, which is often depleted of KCs. A striking difference in the constitutive HBD-2 expression studied is the range of donor differences of expression. KCs taken from juvenile foreskins show a large range of HBD-2 message expression. Donor differences in HBD-2 peptide expression have also been measured in lung epithelia (Hiratsuka, 1999) and may indicate the individuals predisposition to produce different levels of antimicrobial peptides. Consequently, all the alterations in expression measured in these experiments, in response to fungal infection, had to be compared to donor controls.

The stimulatory role of infection and inflammation on HBD-2 transcription has been widely examined. Bacterial pneumonia (Hiratsuka, 1999), cervical infection (Heine et al., 1998) and psoriasis (Henseler and Christophers, 1995) have all been shown to increase the transcription of HBD-2. The skin models selected here also show increased transcription in response to viable *M. furfur* and *C. albicans*, following incubation and this is possibly due to the increase in colonization of the skin model. What is interesting is that epithelia are selective in their response to organisms. *Helicobacter pylori* will only stimulate human gastric cell line HBD-2 expression if it contains the *cag* pathogenicity island; this is a set of genes responsible for pathogenicity which initiate duodenitis, duodenal ulcers and gastric adenocarcinoma (Wada et al., 1999). In addition, the mucoid form of *Pseudomonas aeroginosa* which can cause infection in cystic fibrosis patients, induces HBD-2 expression in lung epithelial cells (Harder et al., 2000) whereas the non-mucoid, non-pathogenic form does not. It is possible that various skin pathogens have pathogen associated triggers which may or may not effect HBD-2 transcription.

These experiments indicate that HBD-2 expression in response to organism challenge, and indeed TNFα exposure is not consistent in skin models, due in part to donor differences in basal HBD-2 expression and in response to inflammatory agents. While certain trends are suggested and some significant results were observed, the lack of correlation between all

donors points to one of two things; either failure in the assay technique utilised to accurately measure alteration in HBD-2 transcription or the inconsistent response of donors to challenge. It is widely known that individual expression of inducible and constitutively produced defensins differs greatly between donors. The response of some skin models to TNFα in these experiments would however indicate a loss of sensitivity of the assay. It is reasonable to assume that the sensitivity of this RT-PCR reaction, combined with donor differences in expression, both contributed to the erratic results obtained in these assays. The lack of general statistical significance in the results negates the possiblilty that any definitive answers may be found using this assay as to whether *M. furfur* or fungal growth affects HBD-2 production. However trends were observed and the results do suggest that *M. furfur* and *C. albicans* growth do stimulate HBD-2 production on the skin models.

It could be hypothesized that an increase in HBD-2 production, although not consistently observed in these experiments, could be due to growth of the organism. An increase in HBD-2 message could be stimulated by the mechanical disruption of corneocytes initiated by growth and, in some instances, hyphal transformation of the organism that has been observed in both LSE and BRT samples under SEM investigation (Figure 87 & Figure 94). *M. furfur* cell wall inactivation of HBD-2 transcription contrasts with the immunogenic capacity of *C. albicans* cell wall at the same concentration. *C. albicans* had previously been reported to have a stimulatory capacity on HBD-2 expression by KC monolayers (Harder et al., 1997), but this is the first time the initiation of HBD-2 transcription has been observed in skin models in response to growth of this organism. Two main hypotheses could explain how *M. furfur* and *C. albicans* could initiate HBD-2 transcription in response to growth in these skin models. Inflammation, as a result of fungal growth could trigger transcription of HBD-2 via a secondary pathway, i.e. via hyphal disruption of the cells which stimulate the cytokine network of the skin, resulting in NFκB activation and HBD-2 transcription. Alternatively, both *M. furfur* and *C. albicans* may possess pathogen patterns, that are recognised by PPRRs of the KC such as the IL-1α R1. No one has yet established a pathogen pattern that acts as a ligand for the IL-1α R 1, but perhaps like other TLR4s, the IL-1R1 can be directly induced by the specific antigen of some pathogens, thus directly stimulating NFκB activation. Hyphal production by both *M. furfur* and *C. albicans* would disrupt a wide range of KCs in both the BRT and LSE epidermis, and so release any stored HBD-2 from cells in the upper epidermis while initiating the primary immune response of

the basal KCs. IL-1 α and TNF α secretion from KCs will also stimulate NFκB activation and thus HBD-2 transcription, therefore contributing to the rise in expression. Hyphal transformation would not account for the stimulation of HBD-2 expression on KC monolayers as *M. furfur* does not revert to hyphal transformation in MEM therefore the slight rise in HBD-2 production of the KC must be due to another factor associated with the viable organism. The organism did remain viable in MEM increasing slightly in cell numbers; therefore, the organism will be producing metabolites in this medium while not greatly proliferating. This growth was, however, only measured in the presence of plastic + MEM, not in the presence of KCs + MEM, so the inhibitory effect of any secreted defensin may have affected *M. furfur* growth in the co-incubated cultures. However as mentioned previously a consistent, statistically significant response to *M. furfur* could not be detected and this hypothesis is purely speculative. In addition these results are limited by the fact that only one strain of *M. furfur* and *C. albicans* was used, perhaps limiting the stimulatory capacity of the organism. While both strains were isolated from clinical samples, which would be assumed to be pathogenic, it would have interesting to examine the effect of various strains of the organism in altering HBD-2 production.

Perhaps the most obvious question to ask, when considering the inducibility of HBD-2, is whether it is active in killing *M. furfur*? Unfortunately purified HBD-2 was not available from other sources. In addition, due to monetary constraints, it was not possible to establish any kind of baculovirus expression system to express the gene transcribing HBD-2 and thereby obtain supplies of HBD-2. As a result the antifungal effect of HBD-2 on *M. furfur* growth in *in vitro* models was not investigated. It would have been interesting to carry out growth inhibition assays utilising the broth microdilution method (Nakamura et al., 2000) to at least determine whether HBD-2 has a measurable effect on *M. furfur* growth. The growth of *M. furfur* in skin suggests that while the organism can revert to pathogenicity, the maintenance of the commensal on the skin must require some form of immune evasion by the organism. While these results do not exactly demonstrate immune evasion the trend for *M. furfur* to not stimulate HBD-2 production as quickly as other yeast, namely *C. albicans*, indicates a possible lack of immunogenicity for the resting, non-proliferating *M. furfur* cell.

Aside from refining the quantitative analysis of HBD-2 production, by measuring either

mRNA or peptide production, various research would be appropriate following this work. Firstly it would be necessary to repeat some of the singular donor experiments carried out to determine if the effects observed were a donor specific. In addition it would be useful to elucidate the exact pathways of HBD-2 transcription by KCs. It would be interesting to break down the initiating components of the cytokine network in controlling the rates of HBD-2 expression. The first candidate would of course be IL-1α and it would be easy to apply anti-IL-1α antibody to KCs to measure their HBD-2 response while blocking this ubiquitous inflammatory agent. In addition it would be interesting to utilise antisense HBD-2 in KC/LSE/ BRT to determine the effect of increased transcription on the organism's viability in these skin models.

3 MELANIN SYNTHESIS AND THE SKIN IMMUNE RESPONSE

3.1 INTRODUCTION

Some of the most interesting pathological effects of *Malassezia* infection manifest as pigmentory changes in the skin. PV, SD and folliculitis, initiated by *M. furfur,* can all present with alteration in the pigmentation of the affected skin and the surrounding areas (Faergemann, 1994). This hyper and hypo-pigmentation can be observed in the tissue affected by *M. furfur* and as yet no definitive evidence exists as to how the organism elicits these pigmentory changes. To elucidate a putative role for *M. furfur* in the alteration of melanin synthesis, the interaction of the organism with MCs must be examined.

3.1.1 MELANOCYTES

MCs are the pigment-producing cells of the skin, derived from stem cell melanoblasts (Cramer, 1991). These neural crest-derived cells migrate during embryogenesis to the basal layer of the epidermis and hair follicles (Cramer, 1991). In the epidermis these cells associate at a ratio of 1:36 with KCs, forming a structural association commonly referred to as the 'epidermal melanin unit'. MCs adopt a dendritic morphology in the basal epidermis contributing to the SIS (Le Poole et al., 1993) and in addition they are the source of the pigmentation organelles of the skin, melanosomes. Melanosomes develop from the endoplasmic reticulum of the MC and are membrane-bound organelles that manufacture melanin (Imokawa, 1989). The development of the melanosomes filamentous structures determines the type of melanin the melanosome will produce. There are two main types of melanin synthesised in mammals; eumelanins which are brown-black in colour and the phaeomelanins, associated with red or yellow pigmentation (Jimbow et al., 1979). As the pigment-producing organelle of the epidermis, melanosome distribution and activity is largely responsible for the general pigmentation of the skin. These organelles are distributed to surrounding KCs via the dendritic processes of the MC. When the melanosomes become filled with melanin they become tethered to myoxin filaments and are transported to KCs via a phagocytotic-like process (Okazaki et al., 1976). Although deposition of melanosomes contributes to the basal level of pigmentation in the skin several other factors can influence the rate of melanin production, as well as the movement of melanosomes.

3.1.1.1 PIGMENTATION

Pigmentation in humans can be influenced by several factors. These include:

Melanoblast migration and differentiation from neural crest cells. This process is genetically predetermined and can be affected by any neural tube defects and factors that interfere with the development of MC stem cells. Stem cell factor receptor is expressed in MCs at this time, following activation of the proto-oncogene, c-kit (Geissler et al., 1988). This results in melanoblast sensitivity to stem cell factor which induces the development and migration of the pre-MC (Nataf et al., 1995).

Melanosome formation. Pre-melanosome structural subunits are arranged in the MC and the subunits are further arranged into the melanosomal structure (Lambert et al., 1999). The melanosome structure partly indicates the type of melanin which will be produced and deposited in the melanosome (Setaluri, 2000). Elliptical arrangement of the actin filaments indicates eumelanin synthesis while spherical, globular melanosomes with a granular appearance indicate pheomelanogenesis.

Melanin synthesis. The melanin synthesis mechanism of melanosomes is a complex metabolic pathway that can be influenced by countless molecules (Hearing and Tsukamoto, 1991). The process, as detailed below (Figure 36), can be triggered by many metabolites including cytokines (Imokawa et al., 1992; Morelli and Norris, 1993), growth factors (Halaban, 1991) and UV irradiation (Birchall et al., 1991).

Melanosome transfer. The phagocytic pathway of melanosome transfer is currently subject to much research (Okazaki et al., 1976). It has been demonstrated recently that melanosome transfer to KCs is dependent upon a G protein coupled receptor on the MC membrane, termed PAR-2 (Sharlow et al., 2000). PAR-2 is activated by serine proteases including trypsin and mast cell tryptase, and activation of PAR-2 *in vivo* results in increased pigmentation of the epidermis due to the increased uptake of melanosomes by KCs. In addition blockage of kinesin activity, which is a protein required for microtubule movement of molecules, results in blockage of UV-induced melanosome transfer in MCs (Hirokawa, 1998). The exact pathway for melanosome transport has not yet, however, been fully elucidated.

3.1.1.2 MELANIN SYNTHESIS

The main factor in pigmentation is however the synthesis of melanin, a complex enzymatic process first elucidated by Raper and Mason (1965). In other animals, pigmentation serves several purposes ranging from sexual attraction to social interaction. In humans, however, the main function of melanin is to protect the epidermis and dermis from ionising radiation. Melanogenesis, as detailed in the Raper-Mason pathway, involves the oxidation of monophenol tyrosine or DOPA (3,4-dihydroxyphenylalanine) chrome catalysed by the integral enzyme tyrosinase (Figure 36).

3.1.1.3 TYROSINASE

Central to melanin synthesis is the glycoprotein tyrosinase (del Marmol and Beermann, 1996). Tyrosinase (monophenol dihydroxy-L-phenylalanine oxygen oxidoreductase) is a 60-70kD, copper-containing enzyme, which catalyses both the hydroxylation of monophenols to o-diphenols and the oxidation of o-diphenols to o-quinones. The albino gene (Kwon et al., 1987), TYR in humans, is responsible for tyrosinase transcription and is a 35kbp gene located on chromosome 11q, 14-21 (Kwon et al., 1989). The TYR gene is transcriptionally spliced to remove introns and the 2.4kb mRNA is translated into the active tyrosinase molecule. The active enzyme is the rate-limiting molecule in melanin synthesis and it becomes incorporated into the melanosome during melanoblast development (Beermann et al., 1990). Tyrosinase is transported to the melanosome in vesicles from the golgi apparatus of the MC (Halaban et al., 2000), and although biologically active at this point, no melanogenesis occurs until the vesicle and enzyme reach the melanosome (Imokawa, 1989). Tyrosinase catalyses three stages in melanin synthesis. Firstly, it catalyses the hydroxylation of tyrosine to DOPA, which causes the pH of the cell to rise, initiating the oxidation of DOPA to DOPAquinone. In addition, tyrosinase also converts 5, 6-dihydroxyindole to indole-quinone. Although critical for melanin synthesis by its catalytic hydroxylation of tyrosine to DOPA, tyrosinase expression is not singularly responsible for melanin synthesis. By stimulating tyrosinase transcription. it is evident that upregulation of gene expression, and indeed increased translation, cannot completely account for the dramatic increase in melanin production in response to stimuli. This is due to two factors. Firstly, many molecules which are active in the melanin

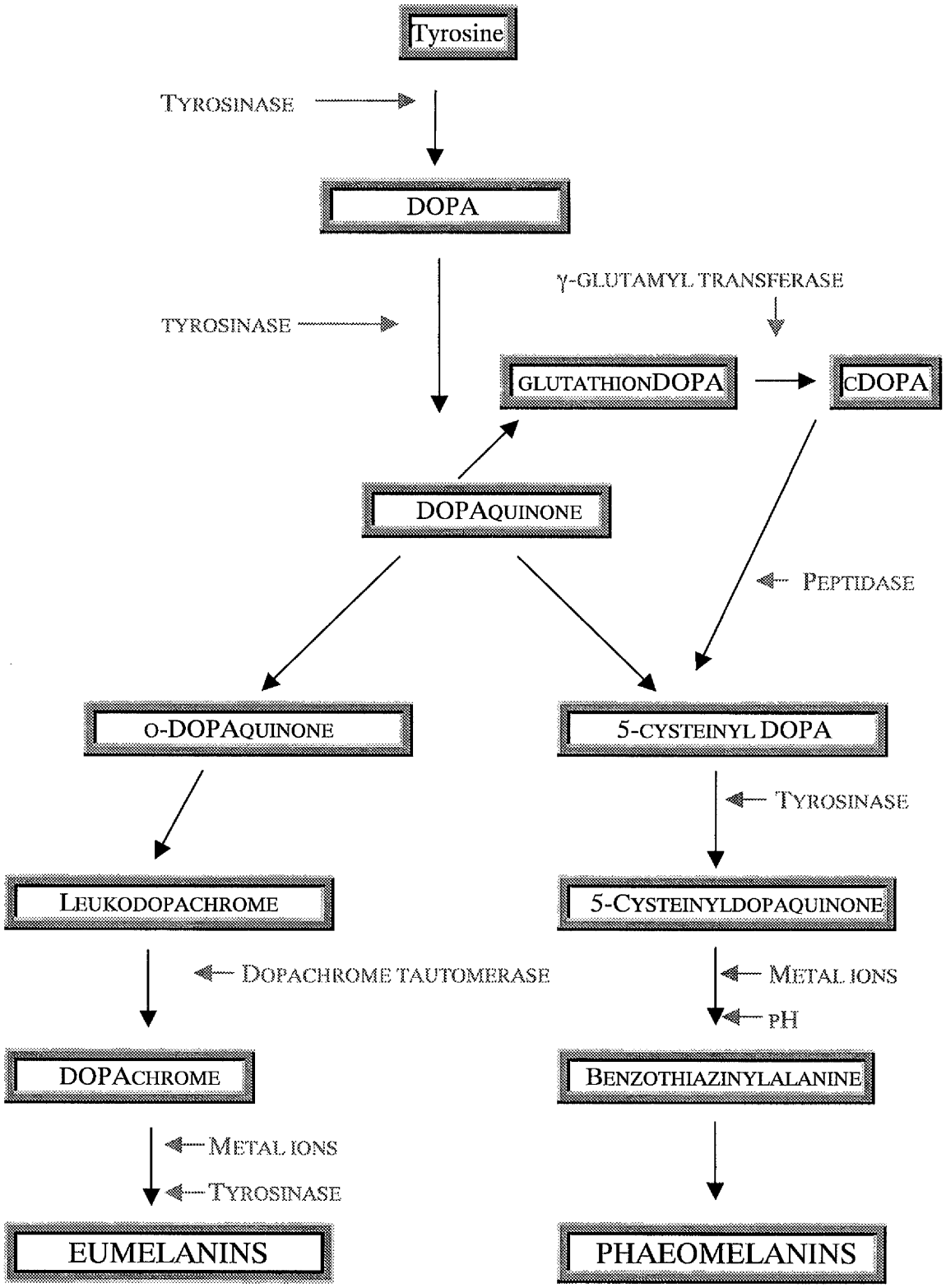


Figure 36: Diagrammatic representation of melanogenesis.

synthesis pathway are inactivated in the melanosome. In addition many other enzymes interact in melanin synthesis and these include the tyrosinase related proteins 1 and 2 (TRP-1, 2) (Sanchez-Ferrer et al., 1995).

3.1.1.4 *TRPs*

TRPs are essential specifically for eumelanin synthesis when, in the absence of thiols and cysteine, DOPAchrome is isomerised to DOPAchrome tautomerase or TRP-2 (del Marmol et al., 1996). The resultant compound is further oxidised by DHICA (5,6-dihydroxyindole) oxidase or TRP-1, to DHICA and this reaction is further catalysed to produce the eumelanins. TRP-1 and –2 genes, located in humans at 9p23 and 13q 31-32 loci respectively, are coordinately regulated with tyrosinase (Kuzumaki et al., 1993). Hence both the TRPs and tyrosinase are essential in melanin synthesis. It has been hypothesised that a plethora of melanogenic inhibitors exist intracellularly which may inhibit tyrosinase synthesis, expression and/or activity. It has been proven that an increase in tyrosinase transcription does not directly equate to an increase in melanin synthesis (del Marmol and Beermann, 1996) and many enzymes are thought to inactivate the already translated tyrosinase. Hence, stimulation of tyrosinase transcription is not fully responsible for melanin synthesis; however, it may induce tyrosinase activity via other important pathways such as increased acidity.

3.1.1.5 STIMULATION OF MELANIN SYNTHESIS

As the main free radical scavenger of the skin, melanin and its production is largely controlled by epidermal exposure to UV irradiation (Johnson et al., 1972). However in addition, melanin synthesis is also affected by inflammatory stimuli and growth factors. These include:

bFGF. Basic fibroblast growth factor (bFGF) has long been recognised as a mitogenic stimulus for MCs (Matsumoto et al., 1991). Melanoma cells have a well documented increase in bFGF receptor expression coupled with increased bFGF intracellular levels (Halaban et al., 1991). In contrast to its induction of mitogenesis in MCs, bFGF does not always stimulate a concomitant increase in melanin synthesis.

IL-1α. KCs produce IL-1α in response to UV (Morelli et al., 1989). This stimulates

expression of high affinity binding sites for α-melanocyte stimulating hormone and this manifests as enhanced cellular responsiveness to MSH with both increased tyrosinase activity and melanin content. Therefore it is possible that IL-1α production may control the reaction of MCs to inflammatory stimuli.

TNF-α. This inflammatory mediator is produced in response to exogenous stimuli from KCs (Strickland et al., 1997) and it is found to inactivate NFκB inhibition. NFκB activation has been shown to down-regulate melanin synthesis in MCs (Englaro et al., 1999) while stimulating the production of cytokines including IL-1α etc. TNFα expression therefore triggers a negative feedback control on melanin synthesis initiated by inflammation.

Endothelin 1. (ET-1) 10μM of ET-1 induces an increase in tyrosinase transcription in MCs coupled with an increase in melanogenesis when secreted from endothelial cells (Imokawa et al., 1992). As ET-1 is secreted by KCs in response to IL-1α, melanin production in the epidermis, particularly in relation to post-inflammatory pigmentation, may depend on secretion of this molecule (Yohn et al., 1993).

αMSH. α-Melanocyte stimulating hormone is a potent inducer of melanin synthesis (Hunt et al., 1994a). Although primarily produced by the pituitary gland, it is also expressed by KCs in response to injury (Schauer et al., 1994), and as such α-MSH is probably the most influential hormone affecting melanin synthesis (Hunt et al., 1994b).

Phospholipase. Secretory phospholipase A2 is the rate-limiting enzyme involved in arachidonic acid liberation from membrane phospholipids (Maeda and Naganuma, 1997). Arachidonic acid is a precursor molecule for many inflammatory mediators including platelet-activating factor and the eicosanoids (Wolf et al., 1986) and it exerts an influence on the melanogenesis of MCs. As secretory phospholipase A2 is released from KCs in response to IL-1α (Birchall et al., 1991) it is thought that this molecule may also be integral to the melanin stimulating cytokine network of the skin.

Histamine. Histamine is a ubiquitous inflammatory factor that induces morphologic

changes and increases in tyrosinase activity in human MCs (Yoshida et al., 2000). These stimulatory effects of histamine are completely inhibited by treatment of the MC with a histamine antagonist, indicating a role for this molecule in post-inflammatory altered pigmentation.

Leukotriene B4. Arachidonic acid and its metabolites (eicosanoids) are membrane-derived inflammatory mediators with a diverse set of biological properties affecting the skin. They have been implicated in the pathogenesis of inflammatory skin disease and post-inflammatory hyperpigmentation. Leukotriene C4 and D4 in particular may affect post-inflammatory MC hyperplasia as they are found to enhance MC growth and melanin synthesis *in vivo* (Morelli et al., 1989).

3.1.2 IL-1α

3.1.2.1 HISTORY

During the 1980s a KC-derived molecule which acted as an inflammatory agent was isolated. This molecule, termed epidermal cell-derived thymocyte activating factor was later identified as IL-1α, a member of the interleukin family of inflammatory mediators. This 31kD factor is expressed constitutively in the basal epidermis, in the absence of exogenous stimuli and can be isolated preformed in the epidermis. In contrast, upregulation of IL-1α translation and biological activity in both the epidermis and KC monolayers is detected in response to trauma, antigen stimulation, UV irradiation, TNFα, TGFβ, CSF and IL-6. The IL-1α content of the skin is therefore controlled by both paracrine and autocrine factors and the regulation of IL-1α in the skin can be considered an axis.

3.1.2.2 IL-1α STRUCTURE

IL-1α is a pleiotropic proinflammatory cytokine secreted by a wide range of cells. The 2 isoforms, α and β, are encoded by two distinct genes on chromosome 2 and they are translated as two 31kD molecules in a range of epithelial, haematopoietic and senescent cells. Although possessing 23% homology, the range of activity and the levels of inducibility of these two cytokines are different. IL-1 α is predominantly secreted, in active form, from epithelial cells while IL-1β is secreted by monocytes, macrophages and antigen-

presenting cells including LCs and other dendritic cells. As IL-1β requires proteolytic cleavage, IL-1α is considered, along with TNFα, to be a primary cytokine in terms of skin immunity and with specific reference to the skin, IL-1α is the main inflammatory cytokine of the IL-1 family.

3.1.2.3 IL-1 AXIS

As mentioned above IL-1α is constitutively expressed by the 'normal' resting epidermis where it does not exhibit an inflammatory effect. The expression and activity of this cytokine is monitored in what has become known as the IL-1 axis (Figure 37)

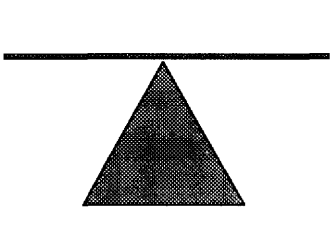


Figure 37: Factors involved in the IL-1α axis

IL-1α exerts its inflammatory effects via an 80kD membrane-spanning receptor termed the IL-1R (type 1). This Toll-like receptor 4 (TLR-4) is activated when its ligand binds to the extracellular portion of this molecule, and to the receptor-associated protein MYD88. This dual binding results in conformational changes in the 215 amino acid cytoplasmic domain and in turn activates the recruitment of a group of serine threonine kinases termed Interleukin receptor-associated kinases. This kinase cascade terminates in phosphorylation of IκBα, which is the ubiquitous NFκB inhibitor, resulting in NFκB-dependent gene expression. The effects of IL-1α and β are therefore monitored by this ligand-receptor binding relationship and two main molecules, the IL-1R (type 2) and IL-1 receptor-antagonist (IL-1ra).

IL-1ra is an 18kD molecule which bonds to IL-1R (1) without initiating signal transduction. It therefore exerts its influence on IL-1α activity by quenching any non-bound IL-1R (1) hence preventing activation of the receptor by IL-1α and β. The IL-1 R (2) works in a similar manner. This 68kD membrane-bound receptor binds IL-1α, β and the MYD88

receptor-associated molecule, and quenches the activity of these ligands by preventing activation of the true receptor. Both of these molecules therefore regulate the effects of IL-1 and indeed their transcription and expression is directly related to the transcription and secretion of IL-1α and β.

3.1.2.4 IL-1 ACTIVITY

IL-1α mediates activity in the skin by initiating a multitude of effects on the cellular components of both the epidermis and dermis. External and paracrine/autocrine stimuli induce release of preformed IL-1α and upregulation of IL-1α transcription. As the primary immune response agent of the skin, IL-1α initiates activation of both the specific and non-specific immune response. For example, IL-1α stimulates IL-8 production from KCs and fibroblasts kick-starting the adaptive immune response, while it also stimulates complement components C3 and factor B release from KCs. IL-1α also induces a range of additional effects which include increased KC proliferation, possibly due to the concomitant increase in growth factor receptor expression; increased ICAM expression in endothelial cells; and chemotaxis of B and T-cells. The multipoietic capacity of IL-1α and the nature of its TLR make it an excellent paradigm of an innate immune response element of the skin.

3.1.3 IL-8

3.1.3.1 INTRODUCTION

IL-8 is a potent neutrophil chemoattractant, classified as a chemokine, which is produced by a variety of cell types upon stimulation. The role of IL-8 in skin disease often focuses upon its involvement in psoriasis and its function, if any, in the innate skin immune response has yet to be defined.

3.1.3.2 IL-8 HISTORY

IL-8 was initially identified in 1988 as an agent involved in controlling leukocyte migration, initially named monocyte chemotactic factor. The family of chemokines that was later identified share common structural and functional homology with IL-8, and currently the human chemokine family numbers in excess of 40 molecules. Chemokines

are small 8-10kD molecules sharing sequence homology based on conserved cysteine residues. They all contain four cysteines that form 2 disulphide bonds and the family is grouped depending on the spacing of the shared cysteine motif (CC, CxC Cx3C). Chemokines exert their influence via the five CxC and eight CC chemokine receptors which exist, primarily on T lymphocytes. The chemokines cross react with each receptor and receptor binding results in activation of cellular immune responses. Chemokine release and binding has been implicated in a wide variety of diseases including HIV infection, where a chemokine receptor functions as a binding site on the T-cell for the virus. In addition, recurrent urinary tract infection associated with dysregulation of IL-8 receptor expression has been identified. In relation to the skin, chemokine expression of IL-8 is shown to be upregulated in psoriasis and is thought to contribute to the T-cell infiltration seen in psoriasis pathology.

3.1.3.3 STRUCTURE/ACTIVITY

Human IL-8 is a dimeric 16kD chemokine of the CxC grouping which is produced by monocytes, epithelial cells and leukocytes. While involved in the regulation of leukocyte maturation and circulation, IL-8 is mainly associated with cellular migration in response to injury and inflammation. As a member of the growth-related superfamily, it shares functional and structural homology with melanoma growth stimulatory activity, GRO-α, β and γ. IL-8 creates a chemoattractant gradient at sites of release and it activates neutrophils, in particular via the CxCR2 receptor on the neutrophil. This results in an increase in cystolic free Ca^{2+} which initiates phagocytosis and superoxide release from the neutrophil. As IL-8 is produced by both KCs and fibroblasts in response to IL-1α (Boxman et al., 1996) and TNFα (Newby et al., 2000) it is thought to be a major component of the cytokine regulatory network of the skin, and it is therefore hypothesized that IL-8 plays an integral role in skin homeostasis and disease pathogenesis.

3.1.4 ENDOTHELIN

3.1.4.1 INTRODUCTION

ETs are a group of three peptides involved mainly in the alteration of endothelial cell adhesion molecule expression and vasoconstriction. ET-1 has been implicated particularly in the dermatological setting, possessing an immunoregulatory function.

3.1.4.2 HISTORY

ET-1 was first recognized in 1988 as the vasoconstrictive component of enriched porcine endothelial cell medium. Subsequently its production has been identified in cancer cell lines, smooth muscle cells, endometrial cells and epithelial cells. In relation to skin cells ET-1 is produced by KCs, FBs and MCs and it effects epidermal cell proliferation in an autocrine and paracrine manner.

3.1.4.3 STRUCTURE/ACTIVITY

ET-1 exerts its influence via binding to the ET-A and ET-B G protein coupled receptors. Binding results in activation of a cellular-specific MAP kinase cascade which stimulates proliferation, and in the case of MCs, melanogenesis. IL-1β, TNFα, TGFβ, UVB and insulin stimulate ET-1 secretion. Secretion of ET-1 from KCs also activates a negative feedback mechanism of ET-1 secretion, via the ET-B receptor.

3.1.5 INFECTION AND PIGMENTATION

It has long been established that inflammation, most commonly resulting from wounding, initiates melanin synthesis in the repaired areas. The role of infectious agents in the alteration of skin pigmentation has not however been examined. While *M. furfur* infection has long been clinically associated with altered pigmentation, little evidence exists as to the mechanism by which the organism elicits an alteration in the normal rate of melanin synthesis. Clinically, PV, an *M. furfur*-initiated infection, presents with scaling and altered areas of pigmentation on the chest and trunk, which has also been identified in penile infection (Smith, 1978). Moderate cellular infiltrates are observed in PV with the majority of infiltrating perivascular cells belonging to the mature T-cell phenotype (Scheynius et al., 1984). Epidemiologically, PV affects 3.1% of the general population (Martins et al., 1989) and *M. furfur* serovar A was found to be the predominant isolate on the chest and back of PV sufferers with no difference in the total population density of *M. furfur* in disease (Ashbee et al., 1993). While no altered levels of overall humoral immunity are detected in PV (Ashbee et al., 1994) 84% of the PV patients' sera recognises a 70 kD component of the oval *M. furfur* exoantigen (Silva et al., 1997). SEM and transmission electron microscopy have been performed on skin material of patients with PV and this has revealed *M. furfur* predominantly in its mycelial phase, inside KCs in this infection. Lipid-like molecules are

detected on the stratum corneum, and inside KCs, which may contribute to the hypopigmentation of some PV lesions, as they may constitute an ultraviolet light block (Borgers et al., 1987).

Gas chromatography-mass spectrometry analyses of *M. furfur* growth on enriched medium demonstrate the generation of linoleic acid hydroperoxides (De Luca et al., 1996). These oxidative agents may utilise skin lipids for ergosterol biosynthesis and as the oxidation of unsaturated fatty acids gives rise to dicarboxylic acids which behave, *in vitro*, as competitive inhibitors of tyrosinase, the lipid metabolism of *M. furfur* may affect normal melanin synthesis in this manner. *Malassezia*-derived lipase also stimulates arachidonic acid release, a precursor for various pro-inflammatory agents from epithelial cell culture (Plotkin et al., 1988), and this cytokine stimulation may be responsible for alteration in melanin synthesis of affected areas. *M. furfur* produces a melanin-like pigmentation when faced with altered nitrogen metabolism (Mayser et al., 1998b); however, the relevance of this study is brought into question by the persistence of pigmented lesions, despite the resolution of infection. It has been shown that *M. furfur* does not directly affect tyrosine metabolism (El-Gothamy, 1981). Hence, the exact mechanism by which *M. furfur* alters melanin synthesis in PV is still unknown. Additional evidence for fungal involvement in melanin production exists, distinct from PV. Anti-MC antibodies are present in the sera of 29% of patients with chronic mucocutaneous candidiasis suggesting that complement-fixing antibodies to MCs are associated with the establishment of chronic mucocutaneous candidiasis (Howanitz et al., 1981).

3.1.6 OBJECTIVES

The purpose of this work was twofold:

• To determine the levels of melanin and tyrosinase expression in skin models, mouse melanoma cells and BRT in response to *M. furfur* and *M. furfur*-derived components.

• To determine the cytokine stimulatory capacity of *M. furfur* and to establish whether there is any difference in donor response to the organism.

3.2 METHODS

3.2.1 TISSUE CULTURE

3.2.1.1 MELANOMA CELLS

B16 F1 mouse melanoma cells (low metastatic rate) were obtained from Dr I. R. Hart, ICRF, London, UK. Cells were seeded at $3x10^3$cells/cm^2 into 35mm petri dishes and incubated in MEM supplemented with penicillin (100 units/ml), streptomycin (100ug/ml), and L- glutamine (2mM) in 5% CO_2 in air for approximately 4 days until confluent. At this time the cells were washed in PBS (3ml) and incubated with 2ml of fungal test suspension (organism, cell wall or cell extract, as detailed previously 2.2.3). Following incubation, cells were washed (2x, 3ml PBS) and trypsinised (0.25% (w/v) trypsin in PBS) for 30s and resuspended in PBS (5ml). Total cell number was obtained by adding 0.5ml of the cell suspension to electrolytic fluid (Isoton 2) for counting using a Coulter counter (Coulter Multisizer 2) thus obtaining the number of cells in the total sample. The cell suspension was then centrifuged (400xg, 10min) and the pellet isolated and assayed for total melanin content.

3.2.1.2 KC MONOLAYERS

KCs were isolated and incubated as previously described (2.2.2.1). When cells reached confluence, monolayers were incubated with serum-free MEM at 37°C in 5% CO_2 in air for 24hr prior to inoculation with 10μg/ml of *M. furfur* and *C. albicans* cell walls (suspended in serum-free MEM as previously described). KCs and cell walls were incubated for 24, 48 and 72hr and following incubation, cell medium was harvested and aliquoted into 300μl samples and stored at –20°C until ELISA could be carried out. Matched KC RNA was isolated as previously described, and estimated by measuring the A_{280}/A_{280} ratio of each sample. RNA was then stored at -70°C till used in semi- quantitative RT-PCR.

3.2.1.3 BRT

BRT tissue was processed, inoculated and incubated as previously described (2.2.1.2). BRT epidermis was isolated by cutting the tissue into 5 x 2mm strips. These strips were then floated on to 0.5% (w/v) dispase in PBS and incubated overnight at 4°C. The tissue

was then placed in a pre-weighed RNAse-free Eppendorf tube and freeze-dried overnight (12hr). Following freeze drying the tissue was weighed and melanin production assayed.

3.2.1.4 BRT RNA ISOLATION

Freeze-dried BRT epidermis was treated as previously described to isolate RNA (2.2.4.1) and RNA integrity and quantity was determined as previously detailed in 2.2.4.1 and 2.2.4.10 respectively.

3.2.2 MELANIN ASSAY

Melanin content was measured by a colorimetric method on a per cell basis. Cells were resuspended in 0.5ml distilled H_2O and following two cycles of freeze/thawing, 0.5ml of 1M perchloric acid was added to the mixture which was then incubated on ice for 10min. Following centrifugation, (300xg, 5min) the pellet was washed twice in 0.5M perchloric acid, 3:1 v/v ethanol : ether and finally with ether. The pellet was dried overnight, 1ml of 0.85M KOH was added, and the mixture was heated for 10min at 100°C. The absorbance of the dissolved melanin was then measured at 400nm and compared against a known standard (Figure 38).

3.2.3 CYTOKINE SEMI-QUANTITATIVE RT-PCR

3.2.3.1 CYTOKINE PRIMERS

Primers for IL-1α, IL-8, ET-1 and GAPDH were synthesised by Gibco, Life technologies (UK) as detailed in Table 18.

3.2.3.2 OPTIMISATION OF RT-PCR

The optimum amplification cycle number for each of the primer sets was established by determining when the amplification curve in the RT-PCR reaction reaches its optimum phase. This was achieved by making a 100μl reaction mixture with each primer set and a constant sample of RNA and analysis of the RT-PCR products (i.e. when cycle number is at an optimum for amplification). Following determination of RT-PCR optimum, 2μl of template KC RNA was subjected to RT-PCR using a Gibco BRL SS-one step reaction as previously described. Following cDNA synthesis (initial incubation of 50°C for 30min and 94°C for 2min) each reaction mixture was amplified by a primer-specific amplification

protocol of:

Denature: 94°C, 15s

Anneal: 55-60°C, 30s

Extension: 70°C, 1min

This cycle was repeated for 33 cycles for IL-1α, 38 cycles for IL-8, 35 cycles for ET-1 and 32cycles for GAPDH to give comparable PCR products.

Table 18: Primers for RT-PCR of cytokines.

Target	PCR Product	Sense primer	Antisense primer	Ref
IL-1α	450 bp	GTA AGC TAT GGC CCA CTC CAT	TGA CTT ATA AGC ACC CAT GTC	(Kondo et al., 1994)
IL-8	250 bp	ATT TCT GCA GCT CTG TGT GAA	TGA ATT CTC AGC CCT CTT CAA	(Redondo et al., 1997)[30]
ET-1	410 bp	TTC CCA CAA AGG CAA CAG ACC G	GAC AGG CCC CGA AGT CTG TCA	(Teraki et al., 1996)
GAPDH	180 bp	TCT GGT AAA AGT GGA TAT TGT TG	GAT GGT GAT GGG ATT TCC	

3.2.3.3 ANALYSIS OF CYTOKINE mRNA EXPRESSION

Having isolated each KC monolayer RNA, mRNA was amplified using IL-8, IL-1α ET-1 and GAPDH primers. 10μl of each separate PCR reaction product was then loaded onto a 1% agarose gel, run at 70 volts for 60min, and was densinometrically analysed using Quantiscan programme as previously described (2.2.4.11). Each cytokine was compared against the intensity of GAPDH-amplified matched RNA, thus allowing a relative measure of expression.

[30] On analysis of genebank data, IL-8 primer proved to be incorrect in paper, ie published as ATT TCA, whereas the mRNA sequence requires ATT TCT for amplification. Author was unaware of the published mistake until this was pointed out.

3.2.4 TYROSINASE QUANTITATIVE RT-PCR

A tyrosinase-specific RNA MIMIC was kindly supplied by Dr Geoff Brownbridge from the Department of Dermatology, University of Glasgow. In brief, the tyrosinase MIMIC (TYRMIMIC) was created by PCR. A segment of neutral DNA (from the pORSVICAT vector) was amplified using composite primers HTYR1M and HTYR2M. During this amplification the HTYR1 and HTYR2 primers were incorporated onto the flagging ends of a 280bp long fragment. The product was then purified using Qiaquick PCR columns (Qiagen) and the concentration determined by the A_{260}/A_{280} ratio.

TYROSINASE PRIMERS

Human tyrosinase mRNA sense primer: **HTYR1 : TTGGCAGATTGTCTGTAGCC**

Human tyrosinase mRNA antisense primer: **HTYR2 : AGGCATTGTGCATGCTGCTT**

TYR MIMIC PRIMERS

Human tyrosinase mimic primer sense:

HTYR1-M1: TTGGCAGATTGTCTGTAGCCTGATATATCCCAA--TGGCATCGTAAAG

Human tyrosinase mimic primer antisense:

HTYR-M2 : AGGCATTGTGCATGCTGCTTTAAGGGTGAACACT--ATCCCATATCACCAG

Cellular RNA was isolated from BRT epidermis and 2µl of template RNA was subjected to RT-PCR using a Gibco BRL SS-one step reaction. In brief, 25 µl of 2x reaction buffer, 1µl of a 10µM primer mixture (HBD-23 and HBD-25), 1µl RT-Taq polymerase, 2µl template and 21 µl RNAse-free H_2O was added to 2µl template RNA. cDNA was initially synthesised by incubating this reaction at 50°C for 30min in a Perkin- Elmer thermal cycler. cDNA was then denatured at 94°C for 30s and optimum amplification was achieved by 36 cycles of

Denature:	94°C, 15s
Anneal:	55-60°C, 30s
Extension:	70°C, 1min

Control PCR, to determine the absence of primer-specific DNA in the RT-PCR reaction, was carried out using a Qiagen PCR as detailed previously (2.2.4.9).

3.2.4.1 GEL ELECTROPHORESIS.

10μl of each reaction was loaded onto 2% agarose (1xTBE buffer with1μg/ml ethidium bromide, as previously detailed, 2.2.4.10). Gels were run at 40 volts for 12hr allowing separation and analysis of the tyrosinase-specific and TYRMIMIC-specific bands which were analysed using Quantiscan as previously described (2.2.4.11).

3.2.4.2 STATISTICAL ANALYSIS/ SAMPLE DETAILS

For the BRT melanin assay at each timepoint three samples were taken for each test substance with half of the tissue used to determine melanin content and the other half used for tyrosinase mRNA assay. The figures are expressed as the mean +/- the SEM. For B16 monolayers each experiment was repeated 3x unless otherwise stated and the mean of these three experiments were expressed as above. Cytokine peptide expression experiments were carried out on 2 KC donors (triplicate samples/test substance/timepoint) while the cytokine RT-PCR assay was tested on 1 KC donor (diplicate samples/test substance/timepoint). Statistical analysis was carried out as previously described (2.2.5).

3.2.5 ELISA

Commercial ELISAs for human IL-1α, IL-8 and ET-1 were obtained from R&D systems, (Oxon, UK) and cytokine measurements obtained as per manufacturers instructions.

3.2.5.1 IL-1α

In brief, for IL-1α assay, cell culture supernatant samples were centrifuged to remove any particulate matter. 200μl of the cell supernatant was added to the anti-cytokine antibody coated wells. Samples were incubated for 2hr at room temperature, aspirated and washed three times with wash buffer. Wells were then incubated with 200μl of IL-1α conjugate for 1hr, aspirated, washed three times with wash buffer and blot dried. Wells were then subjected to colour development by adding 200μl substrate and incubating at room temperature for 20min. Following incubation, 50μl of stop solution was added to each well and the OD of each well measured (3x) at 450nm using a Dynex spectophotometer (Dynex info). When running each sample an IL-1α standard, ranging in concentration from 3.9-250 pg/ml was also measured and regression analysis, using FigP data package, allowed correlation between sample values and standard. The IL-1α assay was repeated three times

and the values were expressed as pg/μg KC RNA[31].

3.2.5.2 IL-8

For the IL-8 assay, 50μl of KC supernatant was added to 100μl of assay diluent and 100μl of IL-8 conjugate and incubated for 2.5hr at room temperature. Wells were then aspirated, and washed six times with wash buffer. Following blot drying, 200μl of substrate solution was added to each well and incubated for 30min at room temperature. 500μl of stop solution was then added and the OD measure at 450nm as above. An IL-8 standard with a concentration range of 31.2-2000 pg/ml was measured during each test and regression analysis, using FigP data package, allowed correlation between sample values and standard. IL-8 measurements were done in triplicate and the values were expressed as pg/μg KC RNA.

3.2.5.3 ENDOTHELIN-1

In brief, 100μl of assay diluent and 100μl of sample or standard was added to each well and incubated at room temperature on a horizontal microplate shaker set at 500rpm for 90min. Following incubation, wells were washed four times with wash buffer and 200μl of ET-1 conjugate was added to each well and incubated at room temperature for 3hr on the microplate shaker. Following incubation, wells were aspirated washed four times with buffer. 200μl of substrate solution was added to each well and incubated at room temperature for a further 30min. The relative light units (RLU) of each sample was determined using a luminometer (Dynex technologies, MLX model luminometer). An ET-1 standard with concentration range of 0.32-5000 pg/ml was measured during each test and ET-1 measurements were assessed in triplicate and sample values expressed as pg/ml.

3.2.6 ORGANISM PREPARATION

3.2.6.1 M. FURFUR

M. furfur was grown and viable cell suspensions were prepared as previously described (2.2.3.1). Crude cell extracts were obtained by inoculating 10ml PBS with a loopful of *M.*

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[31] 200μg/ml of KC RNA = 1 x10^4 cells

furfur (Hook) and emulsifying in a glass universal. This suspension was autoclaved (121°C 12min) and then centrifuged resultant solution centrifuged (400xg, 10min) to remove the cell pellet. The supernatant (10ml) was diluted in MEM and added directly to the cell surface of MCs. Diluted MEM acted as the negative control medium.

3.2.6.2 M. FURFUR CELL WALL

M. furfur cell wall was isolated as previously described (2.2.3.3). Cell walls were diluted in MEM and added directly to the cell surface of MCs or dorsal surface of the BRT.

3.3 RESULTS

3.3.1 MELANIN PRODUCTION BY B16 MELANOMA CELLS

A standard curve of melanin absorption was generated for each experiment. From the standard curve obtained (Figure 38), the measure of melanin synthesis from each sample of cells was obtained via regression analysis of the OD value. Melanin production/ cell in 'normal' untreated B16 cell populations reached a constant level following 72hr incubation after seeding onto 35mm dishes (Figure 39). It was therefore at this time that all experiments were initiated, to negate any possible influence of the fluctuation in melanin synthesis that accompanies growth of the B16 cells.

B16 cells responded to viable *M. furfur* in a dose-dependent manner with a significant increase in melanin production at 72hr (Figure 40). This contrasted sharply with the effect exerted by killed whole *M. furfur* which demonstrated a non significant suppression of melanin synthesis (Figure 41).

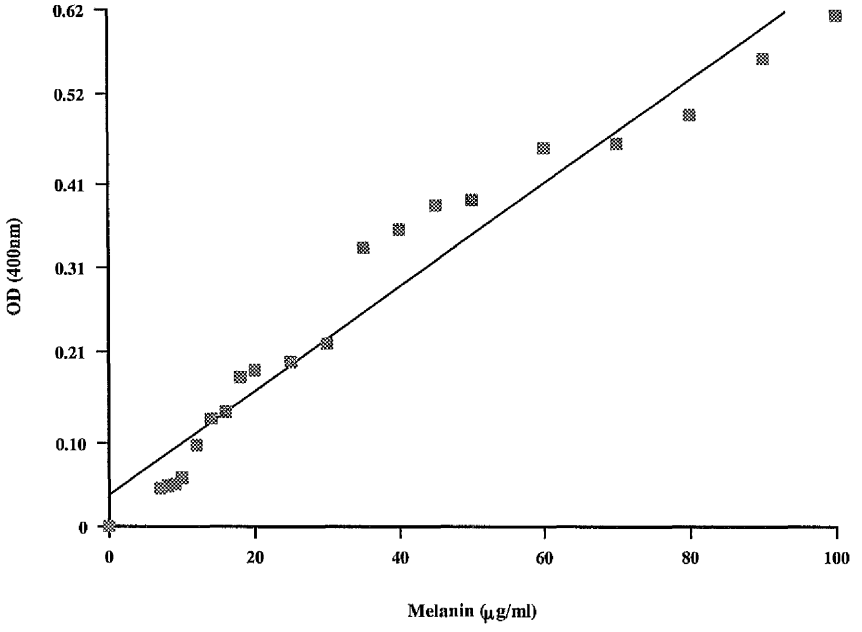

Figure 38: Standard curve of melanin absorbance measured at 400nm.

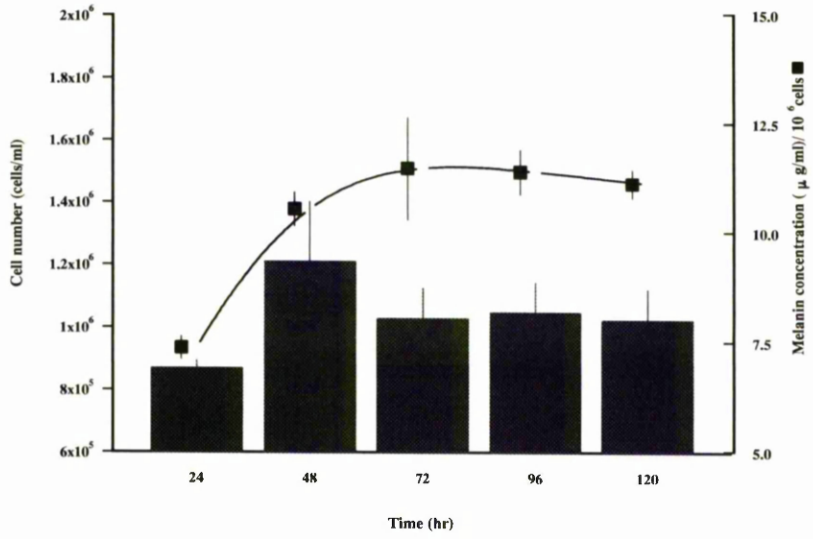

Figure 39: Growth and melanin production by B16 mouse melanoma cells.

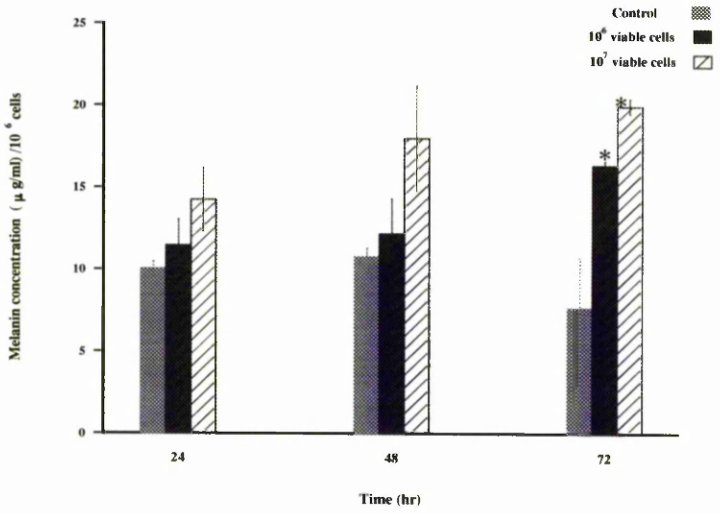

Figure 40: Melanin production by B16 mouse melanoma cells in response to viable *M. furfur*.

* Significant difference from control P<0.01

In a separate experiment the cell wall component of *M. furfur* did not demonstrate inhibition of the melanin synthesis of the B16 mouse melanoma cells (Figure 42) and little difference between the *M. furfur* and *C. albicans* cell wall was observed. The only conclusion which could be drawn from thess data is that *M. furfur* cell wall did not stimulate melanin synthesis of the B16 cells in the same way as viable *M. furfur* did (Figure 40).

To determine which component of the viable cells stimulated melanin synthesis, B16 cells were co-incubated with two dilutions of a crude *M. furfur* cytoplasmic extract (Figure 43). In this experiment the B16 mouse melanoma cell controls exhibited lower levels of melanin synthesis, due to the need to dilute the MEM in PBS to obtain a reasonable control for the extracts. Even though the cells exhibited an overall decrease in melanin synthesis in response to this diluted MEM, it was evident that the concentrated *M. furfur* cytoplasmic extract stimulated melanin synthesis significantly at 48hr as illustrated in Figure 43.

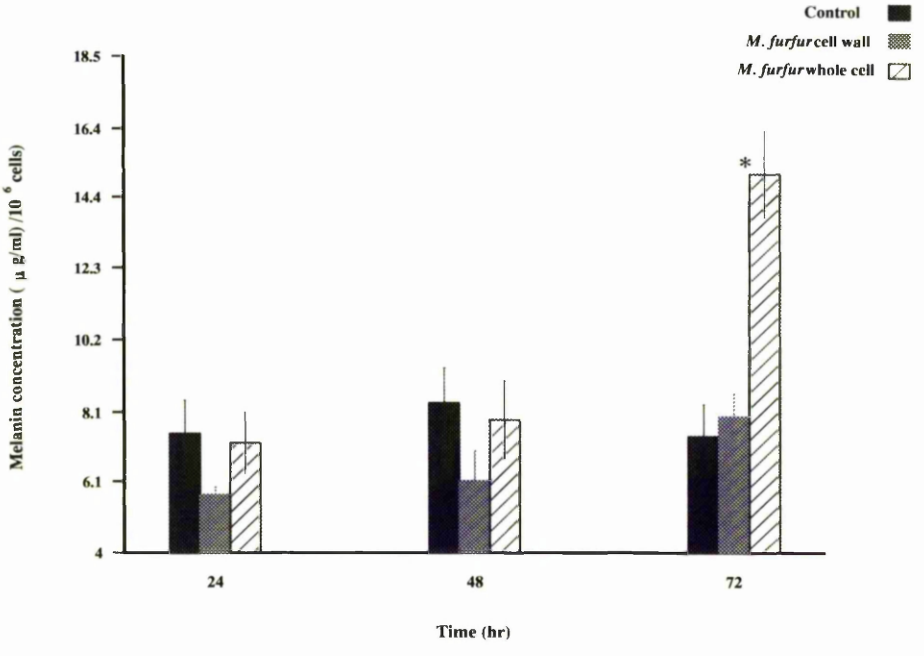

Figure 41: Melanin production by B16 mouse melanoma cells in response to *M. furfur* cell wall (10µg/ml) and killed whole cells (10^6 cells/ml).[32]

* Significantly different to control P<0.01

[32] This figure represents a single assay of B16 melanin synthesis.

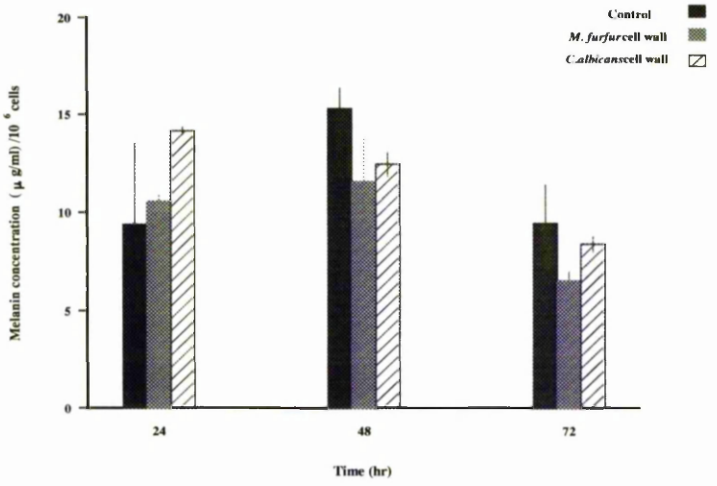

Figure 42: Melanin production by B16 mouse melanoma cells in response to *M. furfur* cell wall (10µg/ml) and *C. albicans* cell wall (10µg/ml) [33].

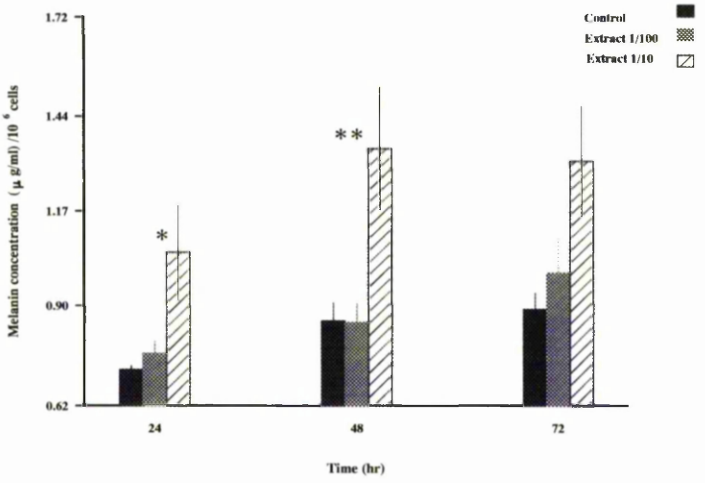

Figure 43: Melanin production by B16 mouse melanoma cells in response to *M. furfur* cytoplasmic extract.

* Borderline significance P>0.01<0.05

** Significant P< 0.01

[33] This figure represents a single assay of B16 melanin synthesis.

This increase in melanin synthesis in the presence of cytoplasmic extract correlated with the increase observed when B16 cells were co-incubated with viable *M. furfur*. However, this was not a *M. furfur*-specific effect as viable *C. albicans* exhibited a similar pattern of stimulation when co-incubated with B16 cells (Figure 44); and indeed, *C. albicans* was more stimulatory than the viable *M furfur*. As the intensity of stimulation was greater with *C. albicans* than *M furfur*, a yeast-associated stimulation of melanin synthesis could be hypothesised. *C. albicans* growth in MEM was rapid compared to *M. furfur* thus the comparison of viable *M. furfur* and *C. albicans* has to be questioned. Although melanin synthesis was expressed as concentration (μg/ml)/10^6 cells, there was an increased death rate of the B16 melanoma cells in response to rapid *C. albicans* growth and the resulting cell death may directly affect the surrounding B16 cell melanin synthesis.

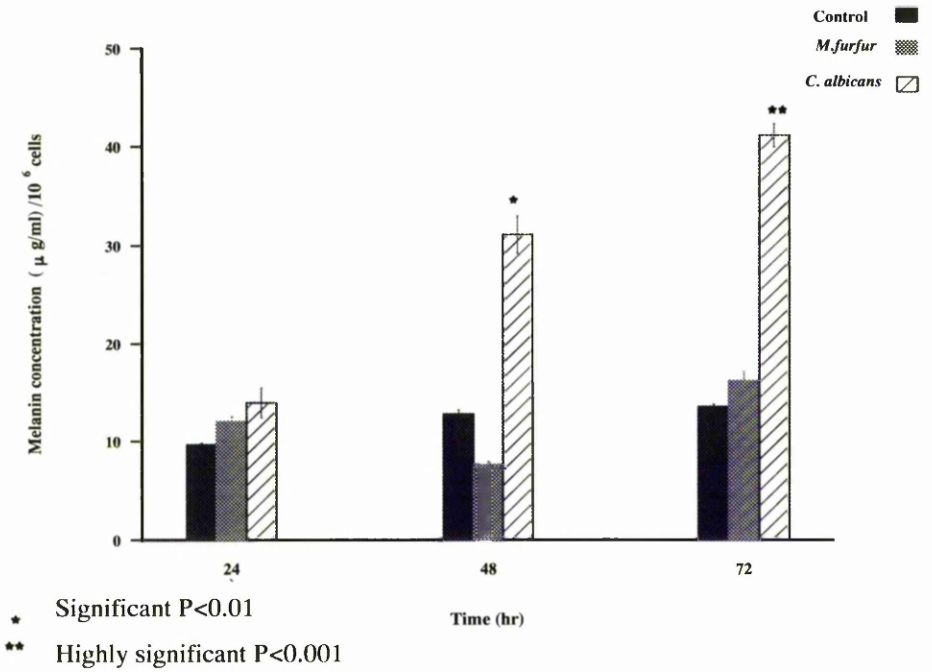

Figure 44: Melanin production by B16 mouse melanoma cells in response to viable *M. furfur* and *C. albicans* (10^6 cells/ml of each).[34]

[34] This figure represents a single assay of B16 melanin synthesis.

To determine the source of melanin stimulation by *C. albicans*, crude cytoplasmic extract was co-incubated with B16 cells. Figure 45 does not demonstrate any '*C. albicans* extract specific' stimulation of melanin synthesis. By comparing the magnitude of melanin expression by B16 cells in response to viable *C. albicans* (Figure 44) and *C. albicans* cytoplasmic extract (Figure 45), it was evident that melanin expression was much higher in response to viable *C. albicans* than it was to *C. albicans* cytoplasmic extract (no difference observed in Figure 45). There was however a correlation between the B16 cell response to viable *M. furfur* and *M. furfur* cytoplasmic extract indicating that viable *C. albicans* may initiate an increase in melanin synthesis, not because of a metabolic by-product (cytoplasmic extract), but because of its direct toxicity as a result of its increased growth rate in MEM, on the B16 cell monolayers. Thus the increase in B16 melanin synthesis in response to viable *C. albicans* may be due to the surrounding cell death while *M. furfur* stimulation could be due to the organism's metabolism.

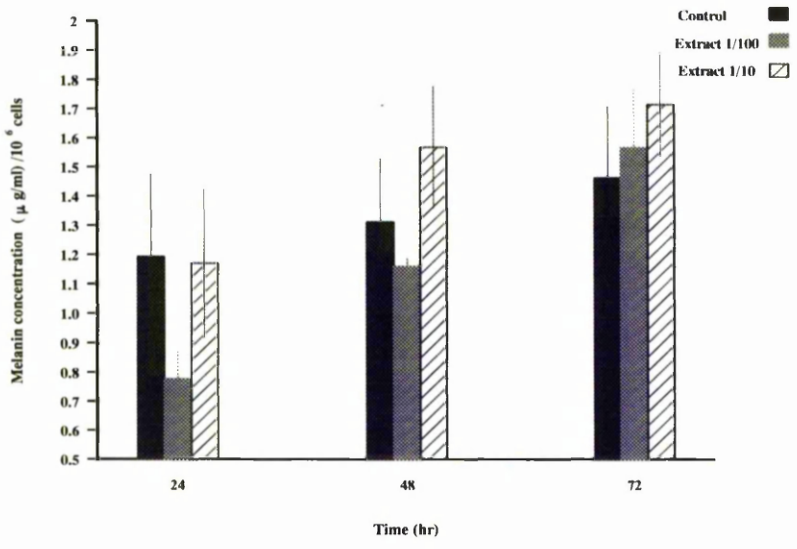

Figure 45: Melanin production by B16 mouse melanoma cells in response to *C. albicans* cytoplasmic extract.

3.3.2 MELANIN PRODUCTION ON THE BRT

Having established that viable *M. furfur* did stimulate melanin synthesis by B16 cells, it was desirable to examine this finding in a skin model system, namely excised BRT. Firstly, on examination of basal levels of melanin synthesis in BRT it was apparent that there were wide donor differences, as indicated by the large error bars in Figure 46. In addition, an increase in melanin production by the BRT was measured over incubation. Thus any increase or decrease in melanin synthesis had to be measured directly against time and donor matched controls.

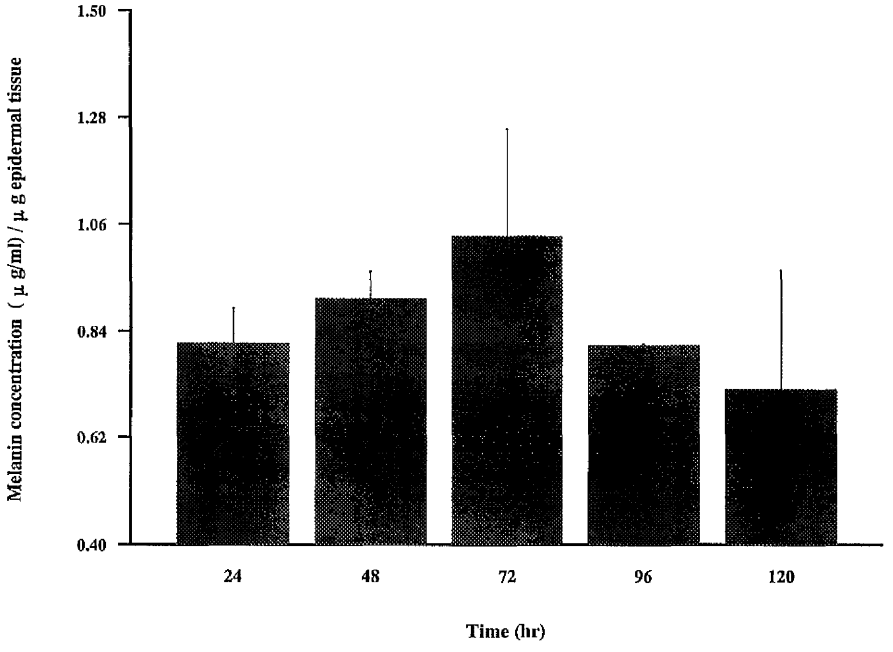

Figure 46: Melanin production by the BRT in response to time, average of 4 donors.

BRT was inoculated with viable *M. furfur* and *C. albicans* and donor differences in the response of the BRT to this experimental infection were detected. Occasionally an increase in melanin production was observed when viable *M. furfur* and *C. albicans* were applied to the dorsal surface of the tissue. As can be seen with donor 18 (Figure 47), there was not a constant increase in melanin synthesis in response to colonisation, since at 48hr a decrease in melanin synthesis was detected in response to both organisms. By contrast, with donor 19 (Figure 48) there was increased melanin synthesis until 72hr when a suppressive effect became apparent. More surprisingly with donor 20, *M. furfur* initiated a larger increase in melanin production than *C. albicans* at 24hr indicating possible 'donor sensitivity' to *M. furfur* growth. With donor 20 (Figure 49), there was an unusual response to *M. furfur* growth at 72hr, with apparent inhibition of melanin synthesis.

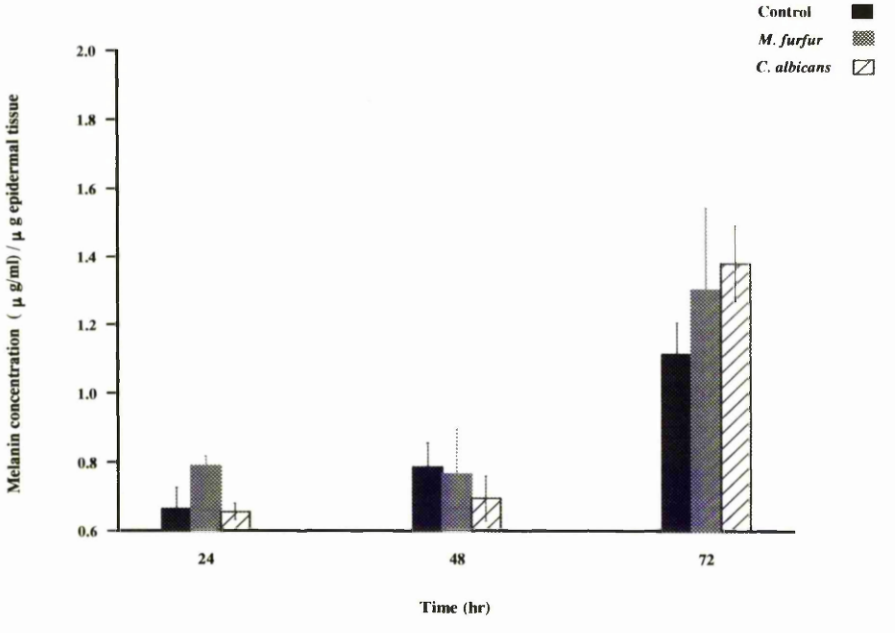

Figure 47: Melanin production by BRT, donor 18, in response to *M. furfur* and *C. albicans* growth.

Table 19: Melanin production [melanin concentration (µg/ml) /µg epidermal tissue] by BRT in response to viable *M. furfur* and *C. albicans*. Range of expression between 3 donors.

	Time (hr)								
	24			48			72		
Donor	Control	*M.furfur*	*C.albicans*	Control	*M. furfur*	*C. albicans*	Control	*M.furfur*	*C.albicans*
18	0.664	0.789	0.656	0.786	0.765	0.694	1.114	1.302	1.382
19	1.121	2.079	1.631	0.937	1.609	1.988	1.635	1.141	1.283
20	0.877	1.373	1.839	0.863	0.955	1.550	2.322	1.008	2.097

Table 20: Tyrosinase production by BRT in respons to viable *M. furfur* and *C. albicans* (Log 10 molecules/µg RNA). Range of expression between 3 donors.

	Time (hr)								
	24			48			72		
Donor	Control	*M.furfur*	*C.albicans*	Control	*M. furfur*	*C. albicans*	Control	*M.furfur*	*C.albicans*
18	11.976	12.305	12.923	12.943	13.695	13.928	13.957	12.961	13.507
19	16.268	16.692	16.968	16.679	16.597	16.987	16.591	15.972	15.577
20	10.818	11.149	11.149	10.896	11.442	11.325	10.957	10.751	10.708

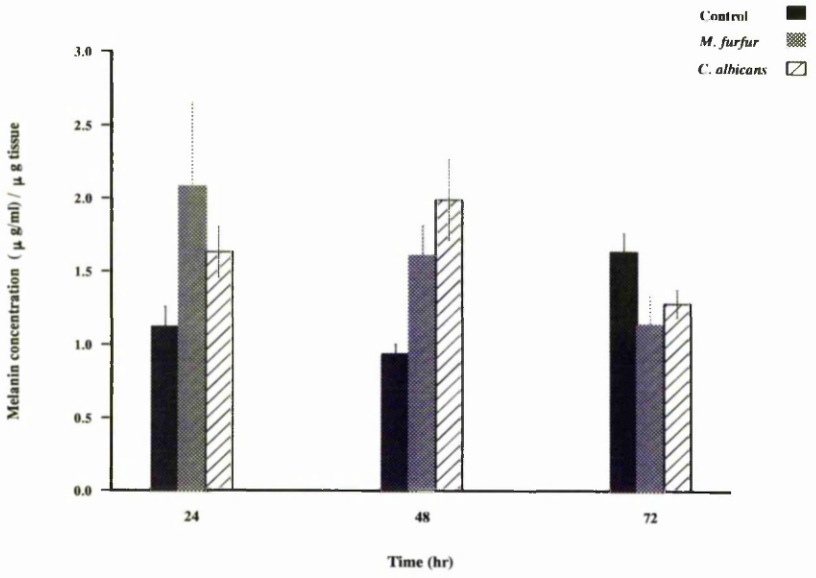

Figure 48: Melanin production by BRT, donor 19, in response to *M. furfur* and *C. albicans* growth.

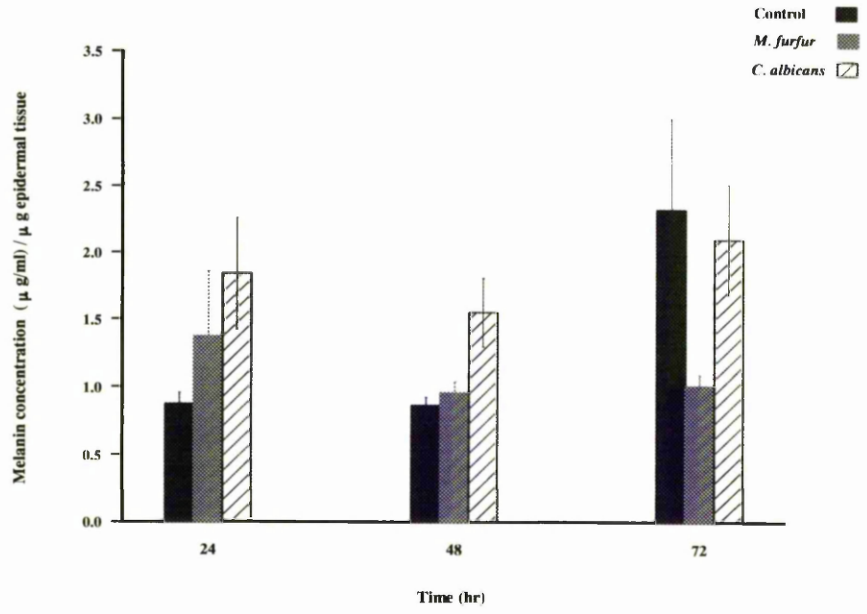

Figure 49: Melanin production by BRT, donor 20, in response to *M. furfur* and *C. albicans* growth.

By examining and comparing these results in Table 19 it was evident that melanin synthesis in response to *C. albicans* and *M. furfur* was not consistently measured in this assay. In addition none of these results demonstrated statistical significance, hence the effectiveness of this assay to measure melanin production on the BRT was questioned. Melanin production was largely unchanged by the addition of yeast cell walls to the BRT (Figure 50). In conclusion little difference in the production of melanin between inoculated and control tissue was observed.

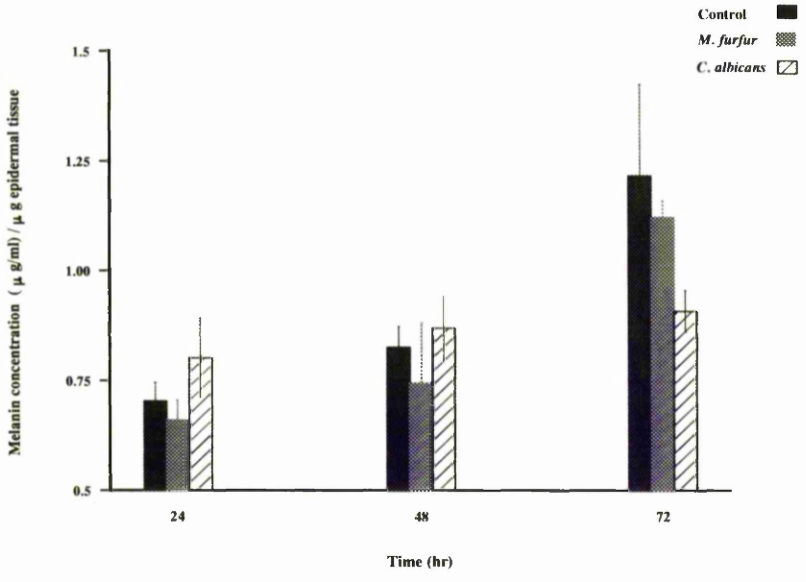

Figure 50: Melanin production by the BRT, donor 21, in response to *M. furfur* and *C. albicans* cell wall (10µg/ml)

3.3.3 TYROSINASE PRODUCTION BY BRT

After verifying the amplification profiles of tyrosinase and the TYRMIMIC in RT-PCR, tissue from 4 donors (which was 'control' incubated over 72hr) was assayed for melanin synthesis and tyrosinase production. From this matched tissue, tyrosinase expression was observed to increase roughly in line with melanin production. However as can be seen in Figure 51, 48hr value, tyrosinase synthesis did not always match up to melanin synthesis.

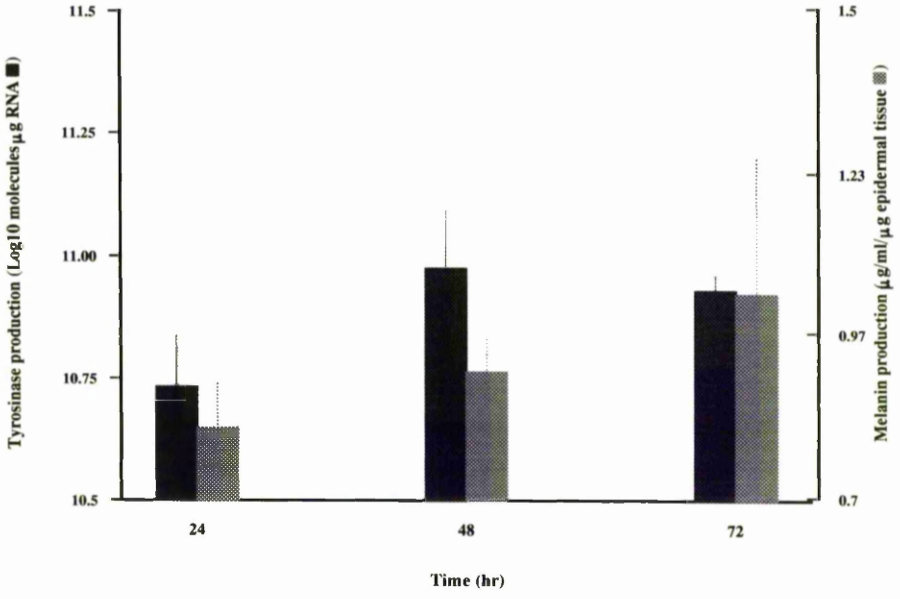

Figure 51: Tyrosinase mRNA and melanin production by the BRT in response to incubation (average of 4 donors).

In general tyrosinase transcription did not equate to the measured melanin synthesis when comparing the two models and as can be seen by comaring these matched tissue, only rarely was a direct correlation observed. As can be seen from Figure 52 and Figure 47 no correlation between tyrosinase transcription and melanin synthesis can be detected. Again with donor 19 (Figure 48), it was not possible to directly correlate tyrosinase transcription to melanin synthesis, in particular with reference to the response at 24hr to *M. furfur* growth (Figure 53). Again little correlation between melanin synthesis and tyrosinase transcription can be observed with donor 20 tissue (Figure 49 & Figure 54) and donor tissue 21 (Figure 50 and Figure 55).

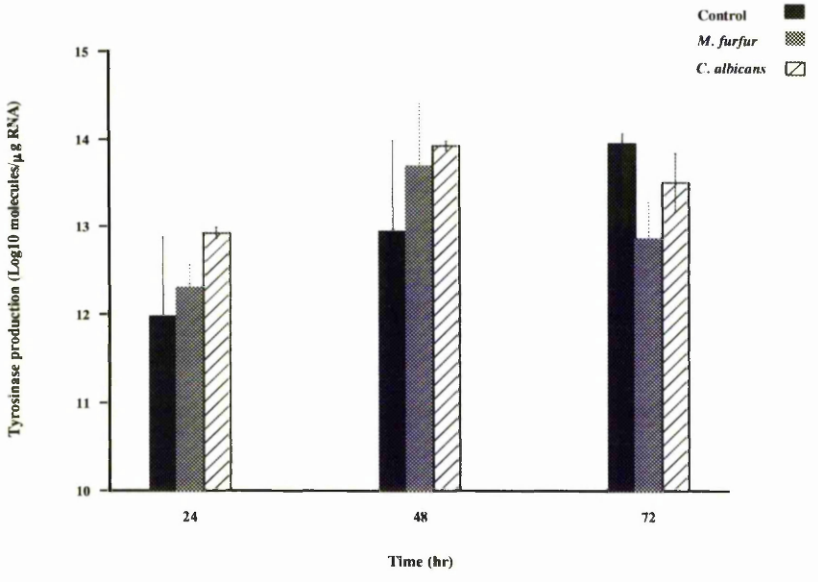

Figure 52: Tyrosinase production by BRT, donor 18, in response to *M. furfur* and *C. albicans* growth.

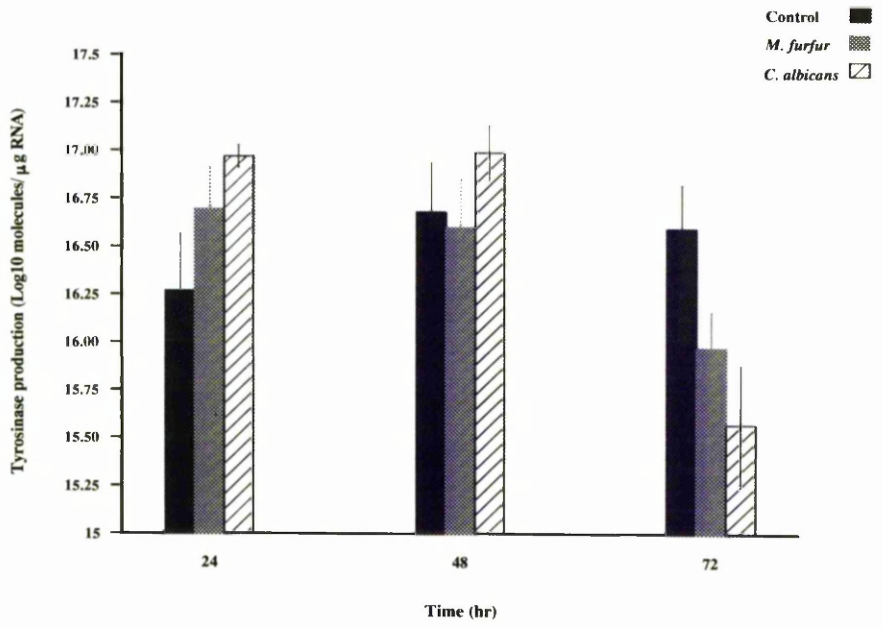

Figure 53: Tyrosinase production by the BRT, donor 19, in response to *M. furfur* and *C. albicans* growth.

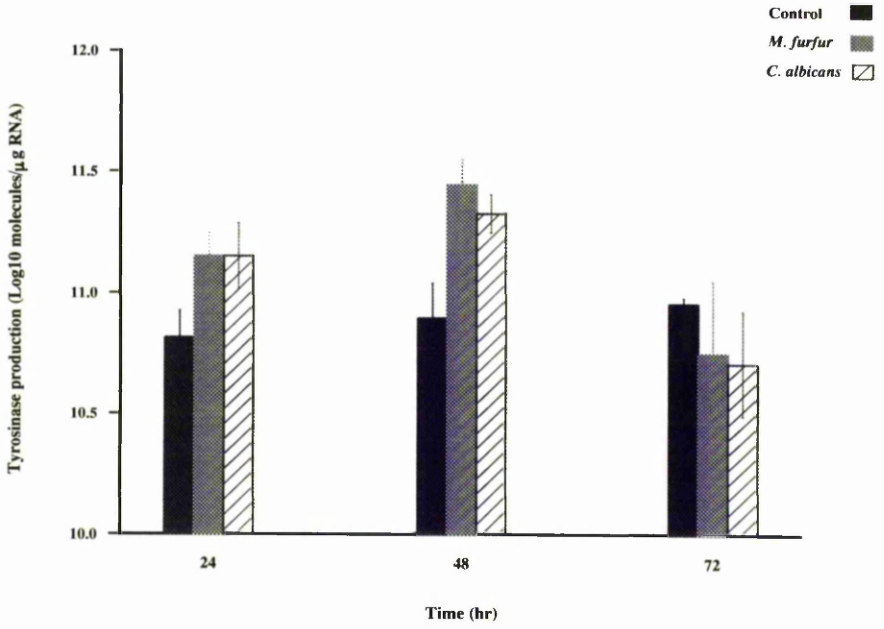

Figure 54: Tyrosinase production by the BRT, donor 20, in response to *M. furfur* and *C. albicans* growth.

In conclusion little evidence of either increased melanin production (Table 19) or

tyrosinase transcription (Table 20), in response to *M. furfur* and *C. albicans* growth, can be gained from these experiments.

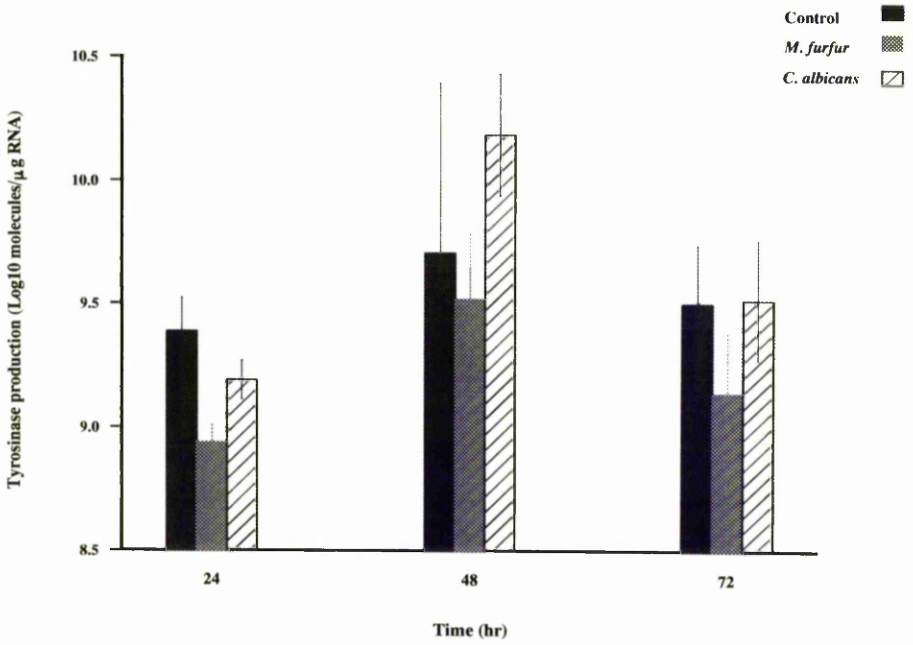

Figure 55: Tyrosinase production by the BRT, donor 21, in response to *M. furfur* and *C. albicans* cell walls (10 µg/ml).

3.3.4 SEMI-QUANTITATIVE RT-PCR

Amplification of KC RNA with appropriate primers allows an estimation of IL-1α, IL-8 and ET-1 mRNA production. The optimum amplification cycle range for each target had to be determined with the optimum number of cycles determined by analysis of the RT-PCR reaction. Figure 56 illustrates the analysis of the RT-PCR amplification of KC RNA for IL-1α, which allowed determination of the optimum cycle number for IL-1α to be 33. Curves for each set of primer in the RT-PCR reaction were also formulated and the optimum cycle number was calculated for IL-8, ET-1 and GAPDH.

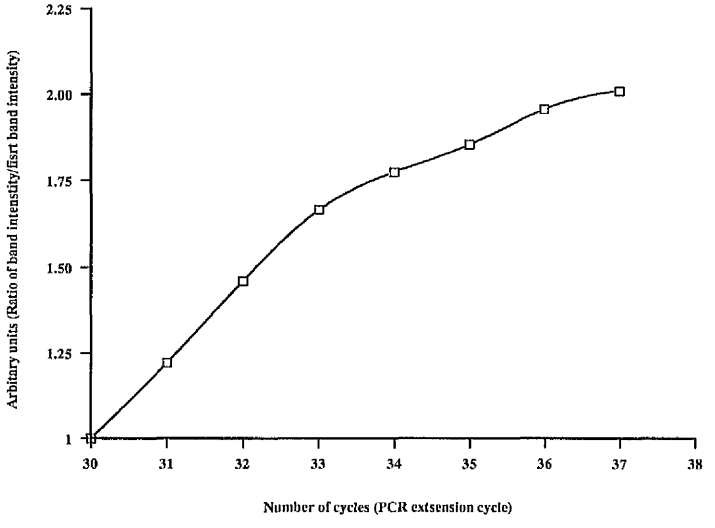

Figure 56: Amplification of IL-α by RT-PCR. Self-limitation of the reaction and the relationship between amplification cycle numbers.

KC monolayers from 1 donor was incubated and tested at 24, 48 and 27hr intervals with the RNA isolated for use in RT-PCR. Statistical analysis could not be carried out on the RT-PCR data due to loss of RNA hence the average value of 2 samples/timepoint/treatment is expressed in Figure 57-Figure 59. As can be seen from Figure 57, there was a general increase in constitutive IL-1α mRNA expression by KC monolayers with culture time and a slight increase in IL-1α mRNA expression in response to TNFα and *M. furfur*/*C. albicans* cell walls was measured. Inhibition of IL-1α expression appeared to be associated with TNFα exposure in this experiment at 72hr however as this experiment was carried out on one donor KC source, with only duplicate samples for measurement, it was impossible to determine if this effect was signifincant. KC monolayer constitutive IL-8 mRNA production could be detected using this PCR, with a decrease in IL-8 production with time (Figure 58). TNFα was particularly potent at stimulating KC IL-8 production at 24hr. However this stimulatory ability decreased with time. *M. furfur* and *C. albicans* did appear to stimulate a slight increase in IL-8 production at 24 and 48hr co-incubation; however no difference in expression was detected at 72hr.

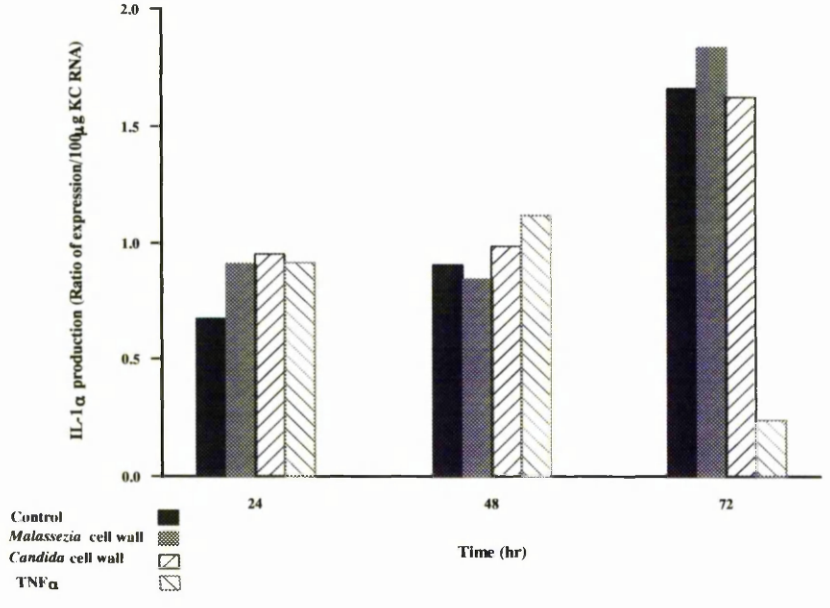

Figure 57: IL-1α mRNA production in response to *M. furfur* and *C. albicans* cell walls (10μg/ml) and TNFα. Semi quantitative RT-PCR amplification of Donor 1 KC RNA.

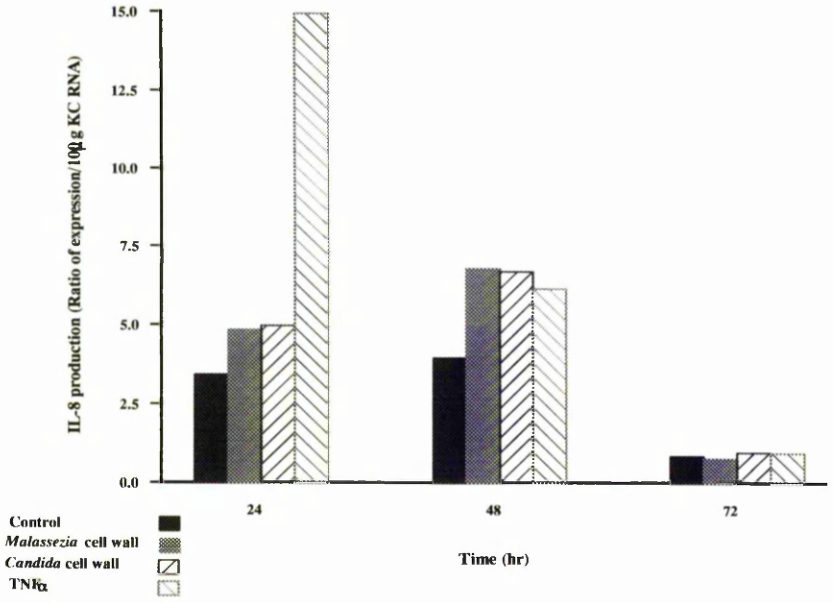

Figure 58: IL-8 mRNA production in response to *M. furfur* and *C. albicans* cell walls (10µg/ml) and TNFα. Semi quantitative RT-PCR amplification of Donor 1 KC RNA.

Constitutive ET-1 production by KC monolayers decreased with incubation time (Figure 59). *M. furfur* and *C. albicans* cell-wall-treated monolayers showed a marked rise in ET-1 secretion at 24hr. No alteration in ET-1 expression could be detected at 48hr and 72hr as a result of exposure to TNFα, *C. albicans* or *M. furfur* cell walls. Comparison of mRNA levels and secreted peptides from IL-1α, IL-8 and ET-1 revealed little correlation. Firstly IL-1α mRNA (Figure 57) and peptide levels (Figure 63) were similar until 72hr. IL-8 mRNA and peptide production (Figure 58, Figure 64) also showed little correlation with some results exhibiting complete disparity i.e. cell wall and TNFα do not stimulate the same increase in message production as they did in peptide production at 48 and 72hr. ET-1 peptide production did not also appear to correlate to message production, with TNFα eliciting a sharp rise in ET-1 peptide production at 24, 48 and 72hr, which was not supported by an increase in ET-1 mRNA synthesis. Due to the sample size (1 donor) and lack of sample repetition this RT-PCR assay proved inconclusive in measuring cytokine mRNA production by KCs.

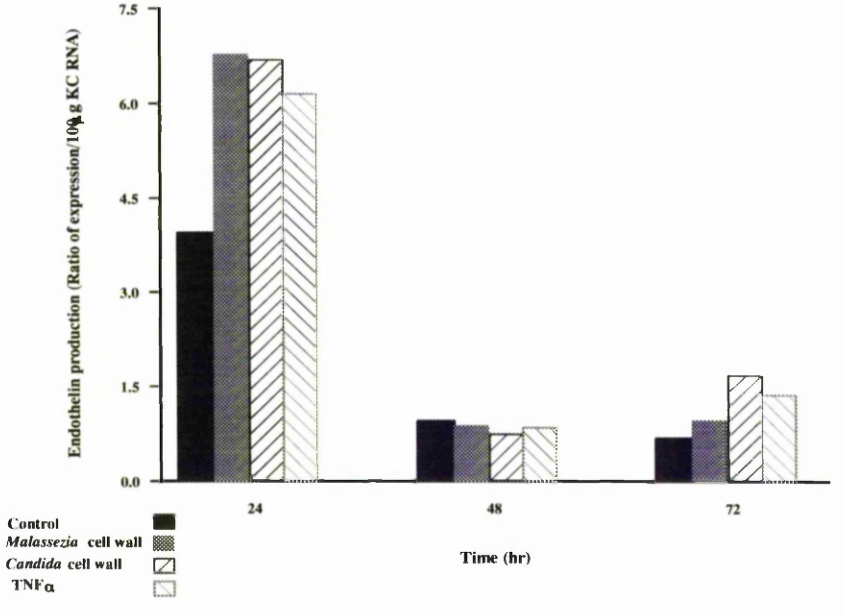

Figure 59: ET-1 mRNA production in response to *M. furfur* and *C. albicans* cell walls (10µg/ml) and TNFα. Semi quantitative RT-PCR amplification of Donor 1KC RNA.

3.3.5 ELISA

Because there was no access to a multi-filtered spectrophotometer, the OD of both the IL-1α (Figure 60) and the IL-8 (Figure 61) samples for ELISA had to be measured without the wavelength correction of 540 and 570nm. The standard curves plotted therefore do not completely correlate to the expected data, as detailed in manufacturers instructions. In addition these ELISAs only maximally achieve 96% recovery of target, however as manufacturers instructions did not comment on the need for correction, the results were not corrected for the additional 4%. ET-1 standard curve was measured usilng a luminometer (Figure 62). Samples were standardized against the total RNA content of each monolayer.

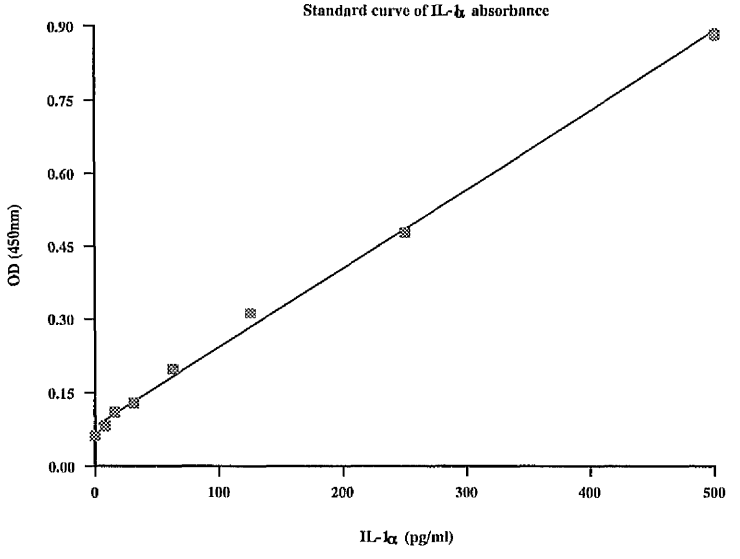

Figure 60: Standard curve of IL-1α absorption at 450nm.

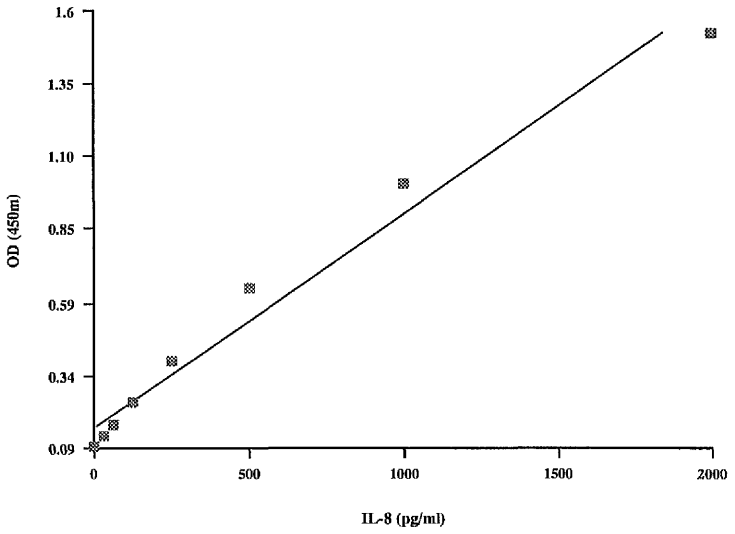

Figure 61: Standard curve of IL-8 absorption at 450nm.

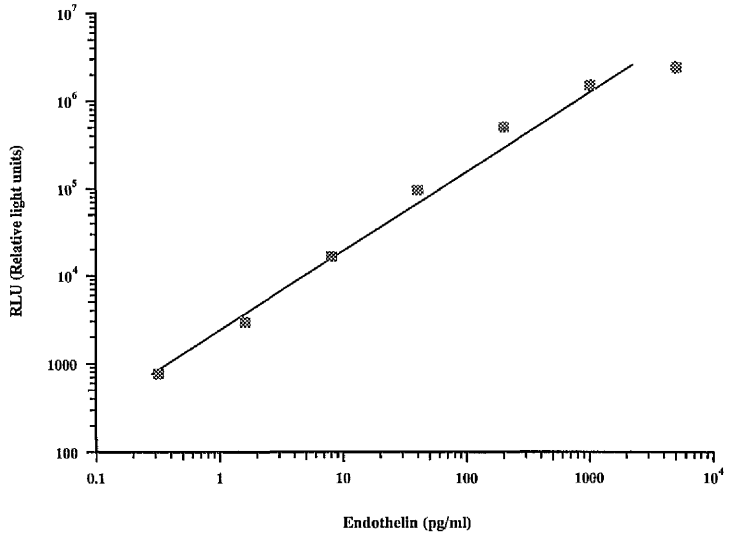

Figure 62: Standard curve of ET-1 luminescence (RLU).

To assay IL-1α levels, 2 KC monolayers were treated with both *M. furfur* and *C. albicans* cell walls (Figure 63- Average of the 2 donors). TNFα was also used as a positive control. No difference in IL-1α production could be detected until 48hr had elapsed. Only then did TNF-α initiate stimulation of IL-1α production, and they coincided with stimulation by *M. furfur* cell wall. At 48hr *C. albicans* cell wall did not elicit an increase in IL-1α production. After 72hr, KC monolayers had produced a significant increase in IL-1α in response to *M. furfur* cell wall, while TNFα had stimulated an even greater increase in IL-1α production at this time. Two interesting points to note regarding IL-1α synthesis are that first constitutive production of IL-1α increases with time in untreated controls. Secondly, *C. albicans* cell wall stimulates an increase in IL-1α production after 72hr.

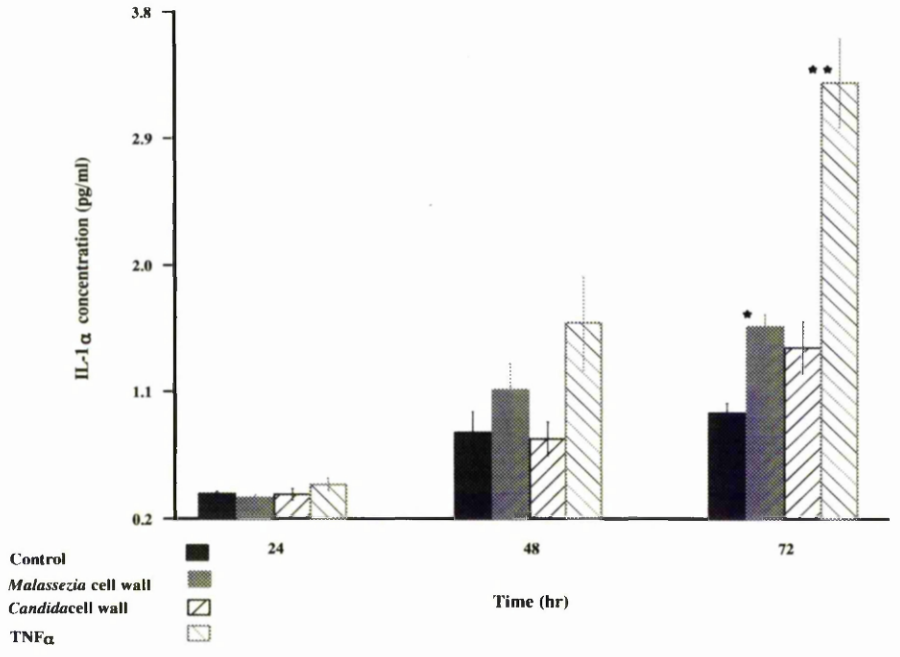

Figure 63: IL-1α secretion by KCs in response to *M. furfur* and *C. albicans* cell walls (10μg/ml) and TNFα.

* Significant difference (P<0.01)

** Highly significant difference (P<0.001)

IL-8 secretion by KCs was also measured in response to co-incubation with *M. furfur* and *C. albicans* cell wall (Figure 64). Constitutive expression of IL-8 by KC monolayers showed an increase in expression with culture time. Positive control TNFα, a potent stimulator of IL-8 secretion, produced a dramatic increase in IL-8 production which dwarfed any change induced by either *C. albicans* or *M. furfur* cell wall; hence these data with TNFα was not shown [35]. Again, *M. furfur* cell wall initiated an increase in IL-8 secretion by KCs at 48hr, while *C. albicans* showed no real increase. This situation was somewhat reversed at 72hr with *M. furfur* cell wall stimulating a further increase in IL-8 production, while *C. albicans* cell wall stimulated a dramatic increase. Again two donors were tested for IL-8 production and comparable results were obtained.

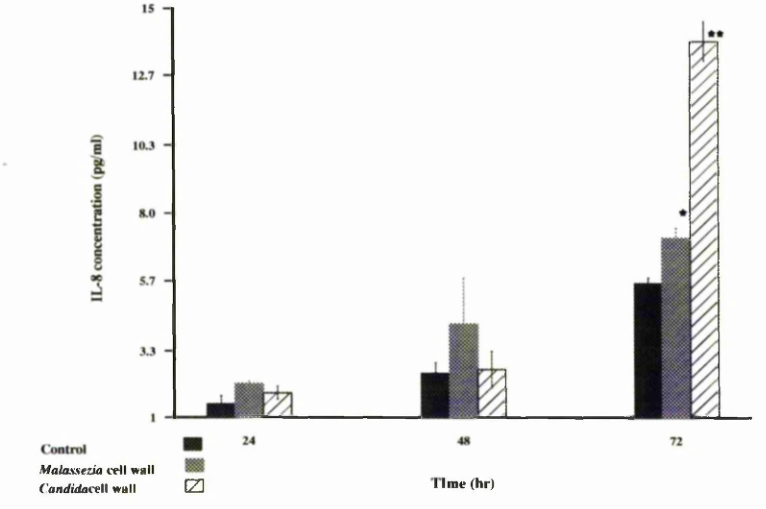

Figure 64: IL-8 secretion by KCs in response to *M. furfur* and *C. albicans* cell walls (10μg/ml).

* Significant difference (P<0.01) ** Highly significant difference (P<0.001)

[35] KC secretion of IL-8 in response to TNFα was highly significant (P<0.001) at all times. Mean production at 24hr was 19.5pg/ml, 48hr 18.6pg/ml and at 72hr 8pg/ml.

The same populations of KCs exhibited a similar pattern of ET-1 expression similar to that for IL-1α, while *M. furfur* and *C. albicans* cell wall did not appear to affect ET-1 synthesis after 48hr (Figure 59). Following incubation for 72hr, *M. furfur* stimulated a rise in detectable ET-1; however statistical analysis revealed that the difference was of borderline significance. What was interesting is that ET-1 production was constitutive and increased with time; moreover TNFα stimulated ET-1 production at every timepoint, without reaching a saturation of stimulation.

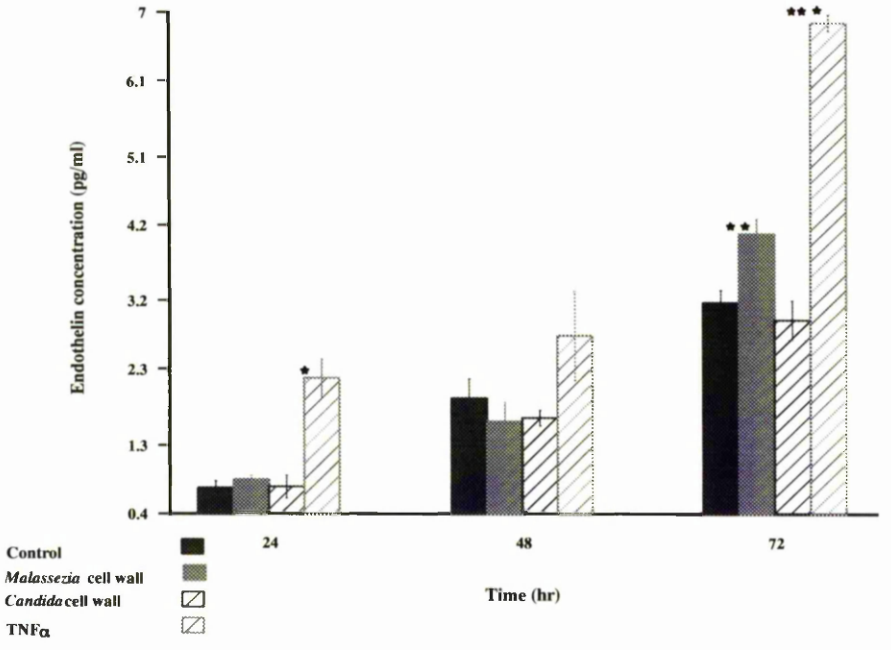

Figure 65: ET-1 secretion by KCs in response to *M. furfur* and *C. albicans* cell walls (10μg/ml) and TNFα.

* Significant difference (P<0.01)

** Borderline significant difference (P>0.01<0.05) [36]

*** Highly significant difference (P<0.001)

[36] Found value of T was so close to the tabulated 'significant' value, with appropriate degrees of freedom, as to warrant comment

3.4 DISCUSSION

3.4.1 MELANIN SYNTHESIS

Melanogenesis in the epidermis is a complex biosynthetic process that centres on the interaction of the epidermal melanin unit, KCs and MCs, with the external environment. Clinically, *M. furfur* infection often presents with altered pigmentation, and following the experimental observation of altered pigmentation in *M. furfur*-inoculated BRT, attempts were made to quantitate the capacity of *M. furfur* to alter melanin synthesis. During growth experiments with *M. furfur* on BRT, changes in the pigmentation of the inoculated tissue were observed. As excised tissue is traumatised and acts very much like wounded skin, an increase in post-excision melanin synthesis would be expected; however inoculated tissue, as compared with donor and time matched control tissue, did demonstrate a trend in increased pigmentation. As *M. furfur* infection often presents clinically with altered melanin synthesis it was decided to investigate the possible pathogen-associated factors which may alter melanin synthesis. The B16 melanoma data suggest that viable *M. furfur* cells could actively upregulate the melanin production in these mouse melanoma cells. It could be argued that these cells of murine origin are not the equivalent of human cells; moreover, the very fact that they are melanoma cells, oncogenic in origin, would point towards their unsuitability in assaying the stimulatory and inhibitory capacity of *M. furfur* in melanin synthesis. However, B16 melanoma cells have been used extensively in assaying the changes in melanin synthesis in response to many chemical and immunomodulatory molecules; this is largely due to their ease of culture and similar melanin metabolism, albeit greatly increased in B16 cells. During these experiments a trend did emerge indicating that *M. furfur* cell walls do not stimulate melanin synthesis whereas viable *M. furfur* cells and cytoplasmic extract appears to have a stimulatory capacity. To determine if *M. furfur* metabolism does alter melanin synthesis these experiments would have to be expanded to investigate the effect of cell wall fractions and metabolites on the HBD-2 production of the cells. The BRT data unfortunately proved inconclusive, and this assay method proved inaccurate in measuring melanin synthesis. As with the HBD-2 assay either the inconsistent nature of BRT donor melanin synthesis or the inaccuracy of the assay itself could be at fault. In this instance I feel that the assay was difficult to perform on BRT with large amounts of tissue required to give a measure of

melanin production. In addition the tyrosinase assay, due to the sensitivity of RT-PCR, was subject to much variation in the results and could not be used to measure melanin synthesis.

Many *M. furfur* agents have been proposed to stimulate melanin synthesis including the cell-associated lipase, which becomes active at pH 7.5 (Plotkin et al., 1996; Ran et al., 1993). Urease, alkaline phosphatase and hydrolase production by *M. furfur* (Dworecka-Kaszak et al., 1994; Mayser et al., 1996), may also contribute to the capacity of the organism to directly affect melanin synthesis. Hence it would be useful to develop a more accurate and reproducible assay to determine which component of the organism contribute to the effect on melanin synthesis see in vivo.

3.4.2 CYTOKINE RESPONSE

The stimulation of the epidermal immune response by *M. furfur*, or the lack of stimulation, is indeed a paradox. While remaining a skin commensal on the majority of the population this lipophilic yeast has been implicated in a wide range of cutaneous infections. Clues as to how the organism elicits hyperproliferation, mild inflammation and altered pigmentation in some individuals may exist in the cytokine immune response of the skin KCs to *M. furfur*. The upregulation of IL-1α in response to *M. furfur* is the first experimental indication that this organism should elicit an innate immune response in the skin. The IL-1R1 is a TLR-4 and these receptors usually only recognize Gram-negative organisms, thereore it is unlikely that yeast would stimulate IL-1α production via the IL-1R1. The induction of IL-1α secretion and transcription may be due to IL-1R1 recognition of lipid moieties of the *M. furfur* cell wall as pathogen-associated molecular patterns and transduces the signal accordingly. Some of the consequences of IL-1α release *in vivo* would account for some of the pathology associated with *M. furfur* infection, including both the hyper- and hypo-pigmentation seen in PV and the increased desquamation observed in SD. In addition to its mitogenic effects on KCs, which could account for this increase in epidermal proliferation, IL-1α also stimulates KC α-MSH release and receptor expression, which could contribute to any increase in pigmentation observed.

As increased IL-1α expression stimulates further cytokine release from the surrounding KCs and epidermal cells, the effect of any initial IL-1α release could stimulate the immune response of these cells, increasing antigen presentation by LCs, stimulating adhesion molecule expression from endothelial cells and amplifying cytokine production via FBs. One of the most interesting results is that despite the stimulatory capacity of *C. albicans* cell wall on HBD-2 production, there appears to be a moderate increase in IL-1α in response to *C. albicans*. As another member of the commensal yeast population, *C. albicans* carriage is common, and the organism often becomes pathogenic only in severe cases of neutropenia and T-cell depletion. Thus the difference between the ability of *M. furfur* and *C. albicans* to initiate disease in 'normal' individuals may lie in the individual's capacity to produce an innate immune response to *M. furfur*. While no donor variation was seen in the trend of the KC response to *M. furfur* cell wall (increase in a time-dependent manner), donor differences in the magnitude of responses was observed. This raises the possibility that some people may experience a more marked response to *M. furfur,* pushing the individual's IL-1 axis over its 'threshold' into action. IL-1α expression will induce ET-1 secretion and the increase in ET-1 expression at 72hr indicates an accumulative effect of IL-1α synthesis on ET-1 expression. ET-1 production is normally associated with endothelial cell vasoconstriction in response to injury; however, KC production may be implicated in both KC mitogenesis and MC melanogenesis. ET-1 stimulates the MC-associated MAP kinases and this phosphorylation cascade terminates in increased tyrosinase production with concomitant increase in melanin synthesis. ET-1 production appears to be stimulated significantly (borderline) at 72hr by *M. furfur* compared to a relative lack of effect by *C. albicans.* This therefore indicates a specific stimulation of ET-1 by *M. furfur* and as such is a possible organism derived mechanism for melanin synthesis. Thus, the rise in ET-1 secretion in response to *M. furfur* cell wall may account for a putative pathway for *M. furfur*-specific induction of melanogenesis.

IL-8 production has been detected in direct response to microbial challenge (Huang et al., 1998). IL-8 is a potent chemokine produced by a wide variety of cells and induced classically by LPS-stimulated monocytes. IL-8 is produced by KCs in response to *M. furfur* cell wall and this result has implications for the pathogenesis of two skin diseases. *M. furfur* has been implicated as an allergen/irritant for both atopic dermatitis and psoriasis. Thus *M. furfur* could act as a primary stimulant in establishing and exacerbating the

dysregulated immune response in psoriasis. The creation of a chemotactic gradient by *M. furfur* could be responsible for the neutrophil and cellular accumulation observed in psoriatic plaques, or it could exacerbate existing plaques. Again, in atopic dermatitis, a positive correlation between head and neck dermatitis and positive patch test for *M. furfur* exists (Kim et al., 1999). The possible IL-8 stimulation in AD in response to *M. furfur* would exacerbate the cellular infiltration observed in AD. It is possible that IL-8 is induced in response to IL-1α induction directly or via the effect of IL-1α on α-MSH stimulation, and its capacity to upregulate IL-8 production. *M. furfur* and *C. albicans* do not necessarily equate to each other in terms of immunomodulating capacity (Huang et al., 1995) and the increase in IL-1α and ET-1 production by KC in response to *M. furfur* cell wall may trigger melanin synthesis in BRT by activating a range of co-factors. Other microbial by-products have been shown to interact in melanin biosynthesis. Staphylococcal exfoliative toxins cleave α-MSH, a potent initiator of MC melanogenesis, and this creates a possible pathway for post- inflammatory hypo-pigmentation following staphylococcal scalded skin syndrome other than the action of its exfoliative toxins.

While only 1 KC donor was used it was evident that cytokine mRNA expression could not be equated to cytokine peptide synthesis using this assay. Little correlation between the mRNA and peptide production. was observed using this particular RT-PCR assay, however as several groups have shown good correlation between peptide and message production then increasing the donor size should allow for correlation of the message and peptide production[37].

[37] Lack of KC donor material prevented repetition of this experiment.

3.4.3 LIMITATIONS OF THE MODELS

There was wide variation in the results obtained from BRT, both in the tyrosinase assay and the melanin assay. This is due to two factors. Firstly, the two assays employed in the tissue assay of tyrosinase transcription and melanin synthesis are very sensitive. RT-PCR, in particular, is extremely sensitive to contamination and can be subject to great variation. In addition, donor differences in the tissue response to the organism exist. BRT, unlike B16 melanoma cells, is not homogenous.

As the method employed previously in this study (Chapter 2) creates a LSE virtually devoid of MCs, this model was not used for any of the melanin synthesis experiments. Recent studies have attempted to standardise the addition of MCs to the LSE so this model could be used for melanin synthesis assays. Adding MCs to the model would allow greater standardisation in the assay techniques employed. For future studies it would be interesting to determine how in a model system more representative of conditions *in vivo*, the cytokines interact in melanin synthesis. This question can only be answered in a co-culture system. It is important, however, to recognise the different KC donor reactions of cytokine production which will still exist on the LSE.

In addition it is evident that there are some limitations to the detection of peptide production by using RT-PCR assays. Firstly trying to assay any alteration in melanin synthesis by measuring tyrosinase transcription indicates the problems in correlating tyrosinase transcription and melanin synthesis. This could be due to several factors, the main one being melanin synthesis does not directly correlate to MC tyrosinase transcription. TRP production, acidity of the melanosome etc all contribute to melanin content, thus tyrosinase transcription is not a direct indication of melanin synthesis.

In summary *M. furfur* appears to have little effect on the melanin synthesis of the skin models examined. While B16 cells exhibit increased melanin production in response to viable *M. furfur*, wide variation in the BRT response was measured. More *in vivo*-like models must be developed to establish if *M. furfur* growth or its cellular components have any effect on melanin synthesis. *M. furfur* and *C. albicans* cell walls did however stimulate IL-1α, IL-8 and ET-1 secretion from KCs as measured by ELISA. This peptide secretion could not be correlated back to transcription using the PCR method employed.

4 GROWTH OF *MALASSEZIA* ON SKIN MODELS

4.1 INTRODUCTION

The likely involvement of *M. furfur* in cutaneous infections has intensified the investigation and experimentation into the growth of this organism. Research has clearly established the dynamics of *M. furfur* growth both *in vivo* and *in vitro* and has enhanced our understanding of the metabolism and distribution of this lipophilic, dimorphic organism.

4.1.1 *MALASSEZIA* POPULATION DYNAMICS

M. furfur can be isolated from the majority of the population following puberty (Faergemann and Fredriksson, 1980). The back and chest are most frequently colonised while areas such as the arms and hands yield fewer organisms (Faergemann et al., 1983c). Between individuals of 30-80 years, there is a declining frequency of colonisation thought to be associated with decreased lipid production (Bergbrant and Faergemann, 1988), which is supported by the yearly decline in colonisation following puberty (Noble and Midgley, 1978). In HIV-positive individuals, some studies indicate a higher density colonisation, however, these increased rates of colonisation do not correlate with increased cutaneous pathogenesis (Pechere et al., 1995). Conflicting reports exist surrounding the role of the declining T-cell population in this increased colonisation rate, with some groups identifying no significant correlation between CD4+ cells (Hakansson et al., 1988) while others express the belief in a connection (Schechtman et al., 1995b). Interestingly, serovar A predominates in the colonisation of the back area; however, no link between serovar type and infection rate exists (Ashbee et al., 1993). Recent studies suggest that it is in fact *M. sympodialis* which is isolated from the back (Aspiroz et al., 1999) while *M. furfur* is isolated from the scalp, possibly due to its lipid preference.

4.1.2 *MALASSEZIA* GROWTH

4.1.2.1 METABOLISM/ ACTIVITY

M. furfur is an obligatory lipophile requiring lipid for growth (Bond and Anthony, 1995), and this is supported *in vivo* where declining lipid isolation from skin correlates with

declining colonisation (Bergbrant, 1991). *In vitro* acid and alkaline phosphatases, phosphohydrolase, leucine arylamidase, beta-glucosidase and lipase production can be detected during growth (Dworecka-Kaszak et al., 1994). As mentioned previously, the production of lipoperoxidase required for ergosterol biosynthesis is essential for growth of *M. furfur* growth (De Luca et al., 1996), and may account for some of the immunological influence of *M. furfur* on skin. Linked to this prerequisite for lipid, *M. furfur* is often isolated from the lipid-rich sites of the host and is frequently implicated in septicemia originating from IV lipid emulsion therapy (Halpin and Dahms, 1983). Unsaturated fatty acids promote growth of *M. furfur in vitro* (Mayser et al., 1995) and another factor which promotes growth of *M. furfur in vitro* and *in vivo* is increased humidity. Occlusion of *M. furfur*-infected sites results in an increased rate of growth of the organism, directly relating to higher levels of trans epidermal water loss, and increased re-infection is observed in geographical areas of high humidity (Faergemann et al., 1983d). Cell wall hydrolase and a hydrolase produced by the metabolizing cell population are thought to be essential in the degradation of skin lipids (Mayser et al., 1996). The skin pH of 5.0 also seems to be the optimum value at which the *M. furfur* lipase is enzymatically active (Ran et al., 1993) and lipase activity is directly related to clinically isolated strains (Riciputo et al., 1996). In systemic infection, *M. furfur* activates the blood coagulation system where the beta-glucan portion of the organism is implicated in clot formation (Suzuki et al., 2000a). *M. furfur* also activates the complement cascade alternatively stimulating production of C5a anaphylatoxin (Terui et al., 1989).

4.1.2.2 Morphology

M. furfur is a dimorphic yeast which exists in oval and hyphal form. *In vitro* cholesterol stearates induce hyphal transformation (Porro et al., 1977) and *in situ* PV +ve specimens exhibit the characteristic 'spaghetti and meatball' hyphae and yeast on KOH digestion of skin scrapings (Abou-Gabal and Fagerland, 1979). Freeze-fracture investigations of PV +ve skin scrapings support the hyphal/yeast morphology in the pathogenesis of PV (Breathnach et al., 1976; Mittag, 1995). Treatment of *M. furfur* with antifungals elicits alterations in the cell wall (McDaniel and Welton, 1984; Moulin-Traffort et al., 1990; Faergemann and Bratel, 1996) due to inhibition of ergosterol biosynthesis by the antifungal. With reference to colony morphology, 3 types prevail, again associated with the areas from which the organism can be isolated (Pechere et al., 1999).

4.1.3 SKIN MODELS

Although skin models are a good representation of skin there are limitations placed upon the uses of such models, because of the morphology of the tissue. The LSE is a three dimensional model of viable stratified epidermis supported upon a FB-contracted collagen matrix. In brief, our protocol utilised collagen obtained from rat-tail tendons seeded with FBs obtained from breast reduction skin. The FBs attach to collagen fibrils and contract down the gel by drawing the fibrils together via contraction of their podia. KCs, obtained from the foreskins of 3-5 year olds, are then seeded onto a submerged collagen matrix, allowed to adhere and form monolayers. When raised to the air/ liquid interface, stimulation of KC differentiation occurs with the formation of distinct basal, spinous, granular and stratum corneum layers. In addition, expression of the appropriate keratin markers is found to be equivalent to *in vivo* levels, and laminin, type IV collagen and β4 integrin are all deposited at the epidermal/dermal junction (Coulomb et al., 1998; Sugihara et al., 1991).

4.1.4 PROLIFERATION OF EPIDERMIS

Hyperproliferation of the epidermis is a characteristic of trauma to the skin, particularly in relation to the immune response. Many factors act as mitogens for KCs; UV, bFGF, IL-8 and ET-1 all stimulate KC proliferation. *M. furfur* infections present with hyperproliferation, ranging from mild in the case of PV, to extreme in SD. Psoriasis, one of the most disturbing hyperproliferative disorders, is a direct response to the abnormal accumulation of immunological factors in the epidermis. Although the disease is genetic in origin, some investigators postulate an abnormal inflammatory response to the normal skin commensal organisms (Bunse and Mahrle, 1996; Elewski, 1990; Squiquera et al., 1994; Xu et al., 1991). AD is also a hyperproliferative disorder of the skin which is linked to *M. furfur* exposure (Broberg and Faergemann, 1995; Broberg et al., 1992; Kieffer et al., 1990; Svejgaard et al., 1989; Waersted and Hjorth, 1985; Wessels et al., 1991). Differential rates of microbial adherence to KCs are not implicated in *Malassezia* infections in the normal host (Bergbrant and Faergemann, 1994) or in the HIV positive population (Schechtman et al., 1995a).

4.1.5 OBJECTIVES

The purpose of this work was twofold;

- To determine the different patterns and rate of growth *M. furfur* and *C. albicans* on the two skin models (BRT and LSE).
- To measure epidermal proliferation in the skin models in response to yeast growth.

4.2 METHODS

4.2.1 TISSUE CULTURE

4.2.1.1 BREAST REDUCTION TISSUE

BRT was isolated and prepared as previously described (2.2.1.2).

4.2.1.2 LSE

LSE was prepared as previously described (2.2.1.1).

4.2.2 ORGANISM PREPARATION

4.2.2.1 M. FURFUR

M. furfur and *C. albicans* were prepared for inoculation onto the BRT and LSE tissue as previously described (2.2.3.1).

4.2.3 GROWTH ANALYSIS

4.2.3.1 QUANTITATIVE MEASUREMENTS

Microorganisms were removed from the BRT and LSE surface, following incubation, by washing the tissue in a sterile universal containing 5ml PBS (230rpm for 1hr). Viability and growth of the organisms were assayed by centrifugation of 4ml of each wash fluid sample (1300xg, 10min) and resuspension of the cell pellet in 400µl of sterile PBS. 100µl of serial dilutions of this concentrated cell pellet were plated onto Dixon's agar, incubated at 37°C for 72hr and a CFU/ml value for the wash fluid was obtained.

4.2.3.2 SEM

Tissues were fixed for SEM staining by staff at IBLS, Univeersity of Glasgow. In brief tissue was fixed in 2.5% glutaraldehyde in 1M Sorrenson buffer[38] for 1hr then washed twice with 0.5M PO_4 buffer. Samples were then postfixed in 1% sodium tetroxide, rinsed

[38] Sorrenson buffer: 9.45g Na_2HPO_4 and 9.07g KH_2PO_4 in 1L distilled H_2O, pH 5.6

once with 0.5M PO_4 buffer and serially dehydrated in graded ethanol (25-100%). Thereafter samples were dried in a critical point dryer, mounted on aluminium stubs, sputter coated with gold and viewed in a Philips 500 scanning electron microscope. Matched tissue was fixed overnight in formalin, stained with PAS and H&E, as detailed in 4.2.4.2 and 4.2.4.3, and a visual observation as to extent of growth obtained.

4.2.4 HISTOLOGY

4.2.4.1 SECTIONING

Following incubation, BRT and LSE tissue was fixed in 4% (v/v) formaldehyde in TBS[39] for 24hr. Tissue was then processed by staff at the Department of Dermatology (Dermatopathology), University of Glasgow, Glasgow. In brief the tissue was dehydrated in graded ethanol, embeding in paraffin wax and cut into 4μm sections using a microtome.

4.2.4.2 HISTOLOGICAL STAINING

H&E staining was carried out on slides by rehydration of the samples in xylene for 5min, ethanol for 3min and H_2O for 1min. Samples were then stained with haematoxylin for 5min, washed in H_2O and alcohol for 5s and in Scott's tap water for 30-60s. Following washing, samples were stained in eosin for 5min, washed, dehydrated and covered with a glass cover slip. Slides were viewed using a Nikon 350 model microscope.

4.2.4.3 FUNGAL STAINING

PAS staining was carried out by rehydrating samples as above, and incubating slides in 0.5% dispase, in PBS, for 1hr at 37°C. Samples were then washed in H_2O for 5-10min, incubated at room temperature in periodic acid for 10min and washed in H_2O. Samples were further incubated in Schiffs reagent for 20min, washed in H_2O and then nuclear staining was carried out by incubating in haematoxylin for 1min. Samples were then washed, rehydrated and covered with a glass slip.

4.2.4.4 IMMUNOHISTOCHEMISTRY

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[39] Tris buffered saline: 900ml saline (8.5gNaCl/L), 100ml tris buffer pH 7.6.

For immunohistochemistry, 4 micron thick paraffin sections were placed on silane-coated slides and fixed by heating overnight at 37°C. Sections were hydrated as detailed above. High-pressure antigen retrieval was then carried out by microwaving the slides on full power (650W) for 25min in 1 litre of 0.25M Tris HCl (pH 7.6), in a pressure cooker. After cooling, slides were left in the buffer for 20min, and then washed in H_2O. Endogenous peroxidase was blocked by adding 0.3% H_2O_2 to each sample and leaving for 15min. Samples were then washed in tap H_2O, left in TBS for 5min, then incubated with 20% normal rabbit serum for 20 min after which the blocking serum was drained off. The sections were then covered with 1/100 dilution of mouse monoclonal primary antibody to Ki-67 in TBS (Ki-67-specific antibody from DAKO Ltd, Angel grove, Ely, Cambridgeshire, UK) and incubated for 60 min at room temperature. Following incubation, samples were washed 3x for 10min in TBS. Samples were then incubated with secondary antibody (biotinylated rabbit anti-mouse,) for 30min, washed in TBS (3x 10min) and incubated with extra-avidin - peroxidase conjugate for 30min. Samples were then washed in TBS (3x over 6min), stained using VIP chromagen kit SK4600 (Vectastain, Vector Laboratories Ltd, Accent Park, Orton, Southgate, Peterborough, UK) and washed in H_2O. Slides were then counterstained in haematoxylin for 2min, H_2O for 1min, acid/alcohol for 30s, Scott's tap water for 1min, H_2O for 5min, methylated spirit for 2min, alcohol for 3min and xylene for 5min and mounted in DPX mountant.

4.2.4.5 PROLIFERATIVE INDEX

A measure of the proliferation of the epidermis was obtained by counting the number of Ki-67-positive cells/ 1000μm of basal epidermis. As Ki-67 is a measure of active cellular proliferation it allowed a comparative estimaion of the proliferation of the skin models epidermis.

4.3 RESULTS

4.3.1 SEM OBSERVATIONS OF SKIN MODELS

The two skin models, LSE (Figure 66 & Figure 67) and BRT (Figure 69), showed similar properties on SEM investigation. The stratum corneum of both models, the main area of colonisation for *Malassezia* species, displayed similar dorsal surfaces. The stratum corneum of LSE however had a slightly higher rate of desquamation as indicated by the increased number of loosened corneocytes observed in SEM (Figure 67). Despite identical methods of treatment during preparation for microscopy, the LSE showed distinct corneocyte shedding following 24hr incubation, whereas the BRT desquamation rate was only comparable with the LSE following 96hr post-excision incubation (Figure 69).

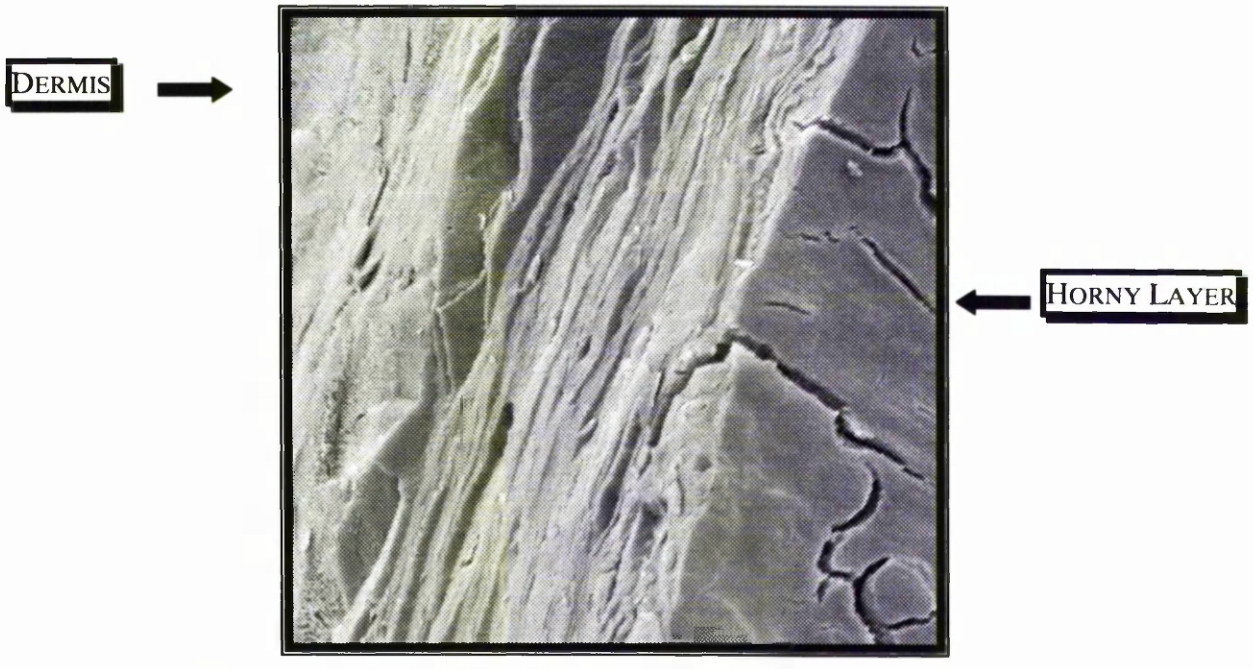

Figure 66: Scanning electron micrograph of transverse section of LSE (x400)

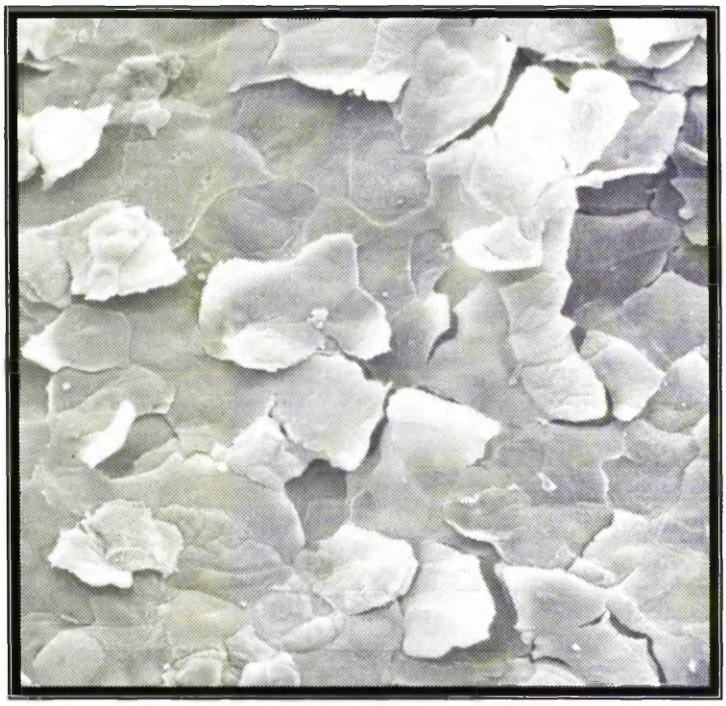

Figure 67: Scanning electron micrograph of the dorsal surface of the LSE following 24hr incubation. Desquamation of epidermis evident (x800).

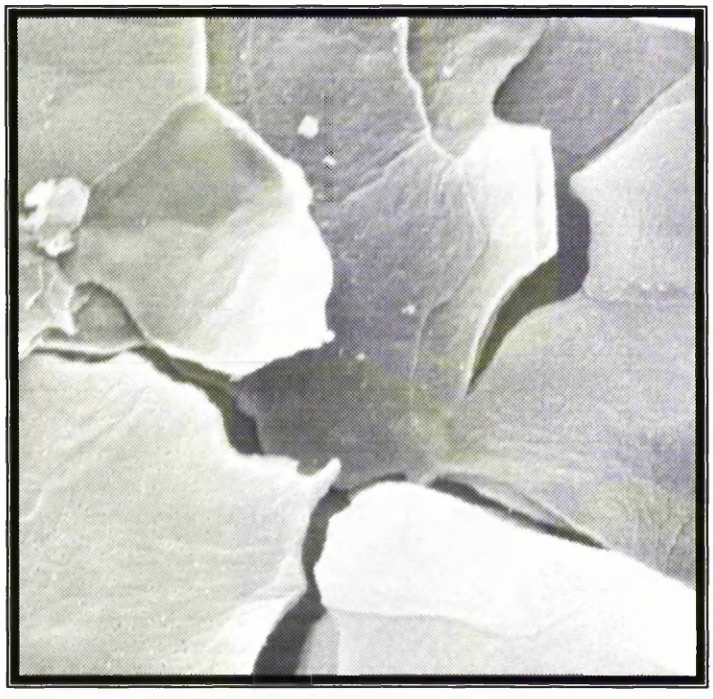

Figure 68: Scanning electron micrograph of the individual corneocytes on the dorsal surface of the LSE (x1200).

Overall the surfaces of both the BRT and LSE and specifically the dorsal surfaces, resembled each other sufficiently well to allow SEM comparison of the growth of *M. furfur* on each. One of the more obvious differences in microscopic evaluation of the tissues was the presence of hair follicles in the BRT (Figure 70). The tissue fixed was approximately $1cm^2$ and hair follicles were commonplace over the dorsal surface.

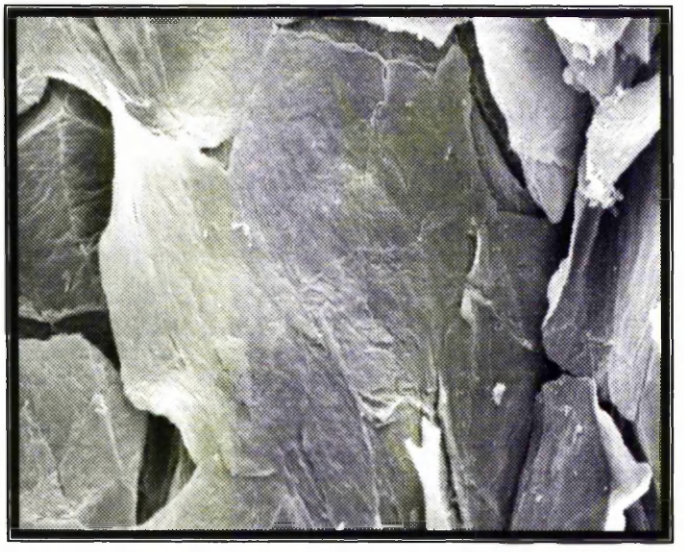

Figure 69: Scanning electron micrograph of the BRT dorsal surface following 96hr incubation. Individual corneocytes evident (x1200).

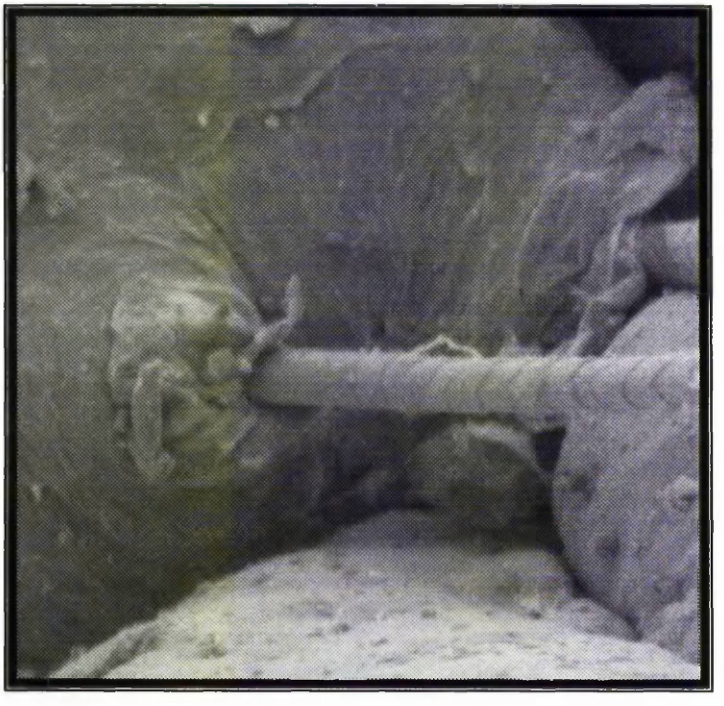

Figure 70: Scanning electron micrograph of BRT with hair follicle (x1200).

4.3.2 SEM OBSERVATIONS OF FUNGAL GROWTH ON LSE

4.3.2.1 GROWTH OF M. FURFUR ON LSE

Although distributed in an identical fashion, *M. furfur* and *C. albicans* had very distinct patterns of colonisation on the LSE. Following 24hr incubation, *M. furfur* was distributed in distinct colonies or clusters (Figure 71). Within these clusters, budding of the organism was limited and there was a discrete border to each colony with large areas un-colonised between these colonies. Figure 71 displays budding cells located on the LSE following 24hr incubation with *M. furfur* at different stages in the organism's life cycle evident. The larger cells are those which are predominantly budding with the seperated smaller daughter cells, surrounding these large cells having become separated previously. Budding was observed frequently at this stage in these isolated colonies. Hyphal production was not observed until 48hr incubation (Figure 72); when the characteristic irregular hyphae of *M. furfur* could be seen on the LSE at 48hr (Figure 73). The hyphae produced by *M. furfur* are wide compared to the mother cell diameter and both Figure 73 and Figure 74 demonstrate this.

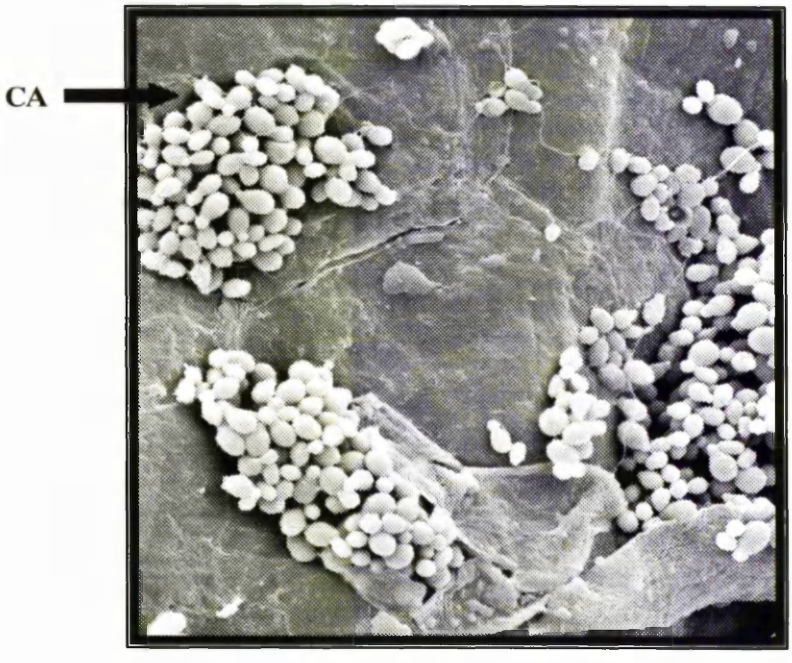

Figure 71: Scanning electron micrograph of *M. furfur* on LSE following 24hr incubation at 37^{0}C. Cavitation of the epidermis (CA) and budding evident (x1200).

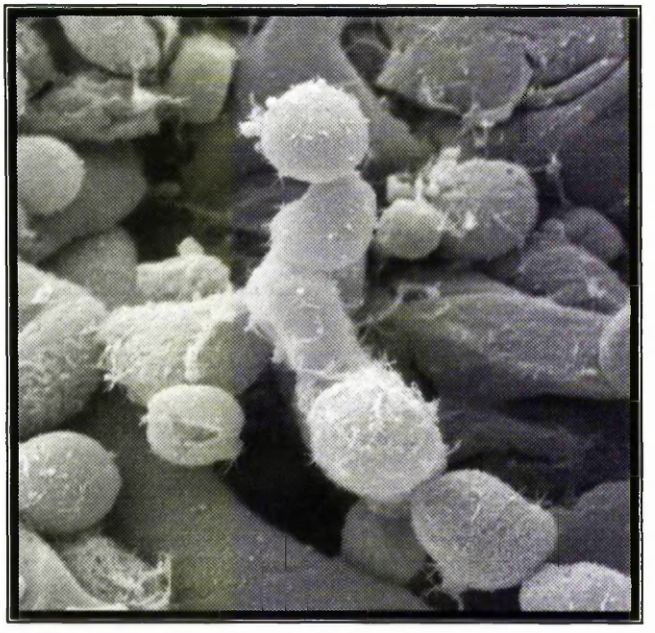

Figure 72: Scanning electron micrograph of *M. furfur* on LSE following 48hr incubation. Hyphal transformation observed (x4000).

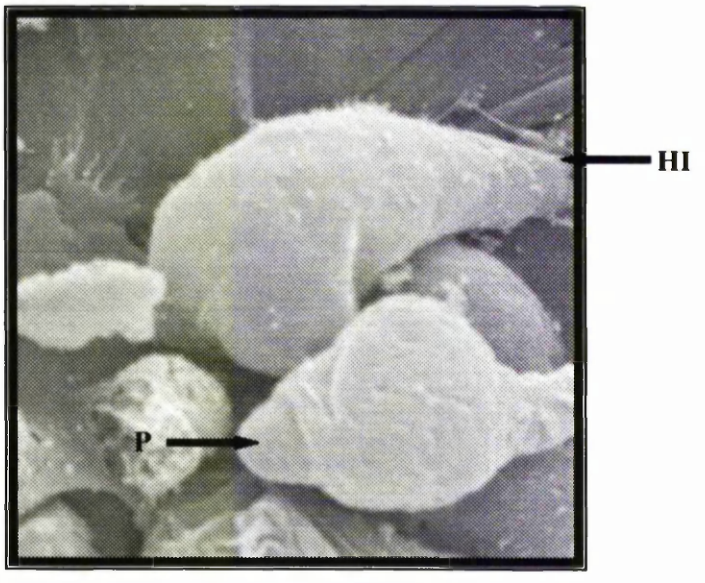

Figure 73: Scanning electron micrograph of *M. furfur* on LSE following 72hr incubation. Philaospore extrusion (P) and hyphal invasion (HI) into stratum corneum observed (x8000).

Figure 73 illustrates the presence of a surface coating on the organism. The coating itself is not uniform and could be a glycocalyx-like material. Figure 73 and Figure 74 shows how the *M. furfur* hyphae breach the stratum corneum, with insertion through a corneocyte being evident. This micrograph supports the notion that *M. furfur* produces a keratinolytic enzyme that aids its invasion and allows it to penetrate the stratum corneum. Figure 74 shows the more widespread production of hyphae following 72hr incubation on the LSE, and again illustrates the insertion of the hyphae into the stratum corneum.

Figure 74: Scanning electron micrograph of *M. furfur* on LSE at 72hr. Hyphal extension and budding evident with hyphal invasion into the stratum corneum (x1200).

4.3.2.2 C. ALBICANS GROWTH ON LSE

Widespread hyphal transformation of *C. albicans* was observed on the LSE following incubation for 24hr incubation (Figure 75) or 48hr (Figure 77). Large areas of the tissue were covered with numerous hyphae and blastoconidia forming a typical biofilm[40] on the tissue surface (Figure 76).

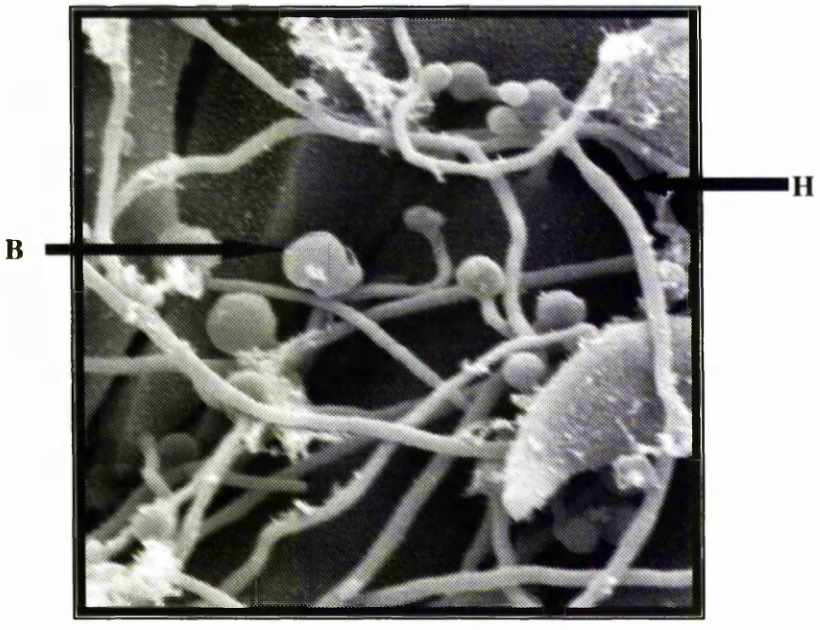

Figure 75: Scanning electron micrograph of *C. albicans* growth on LSE following 24hr incubation. Spherical yeast cells with true hyphae (H) and blastoconidia (B) present (x4000).

[40] For the context of this dscussion a biofilm shall be defined as a collection of yeast/hyphal colonies associated together and attached to the skin surface. This shall include those with or without any extracellular matrix material deposition.

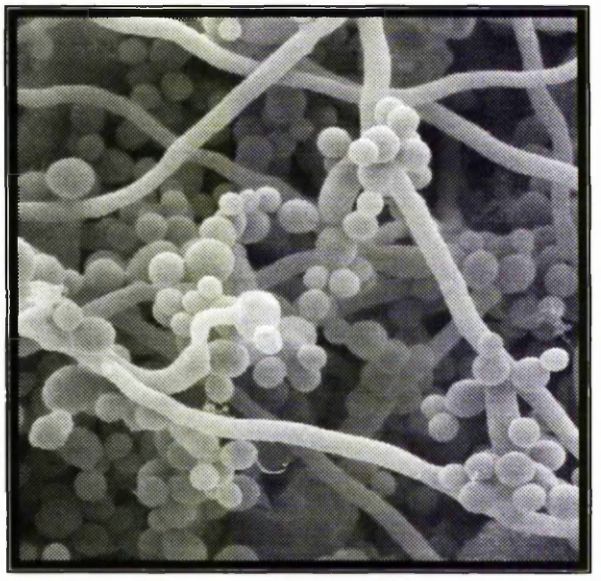

Figure 76: Scanning electron micrograph *C. albicans* on LSE following 48hr incubation (x4000).

Following incubation for 48hr hyphae were frequently observed on the perimeter of the *C. albicans* biofilm (Figure 77 and Figure 78). These hyphae often inserted into the stratum corneum of the epidermis (Figure 78), sometimes resulting in widespread destruction of the LSE surface after 72hr. Closer investigation reveals that true hyphae are produced by the *C. albicans* at 48hr (Figure 79). As can be seen from Figure 80 and Figure 76 hyphae production and branching were so widespread as to create a biofilm effect over the dorsal surface of the tissue.

Figure 77: Scanning electron micrograph of *C. albicans* on LSE following 48hr incubation. Evidence of hyphal invasion into the stratum corneum (x8000).

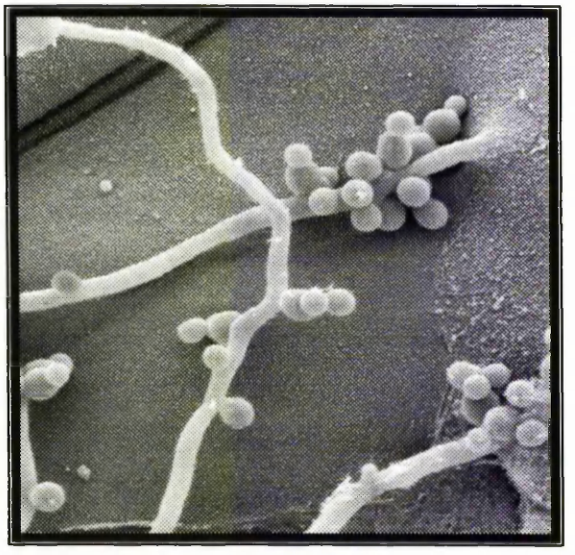

Figure 78: Scanning electron micrograph of *C. albicans* on LSE following 48hr incubation. Conidiophores observed with hyphae and invasion into epidermis (x4000).

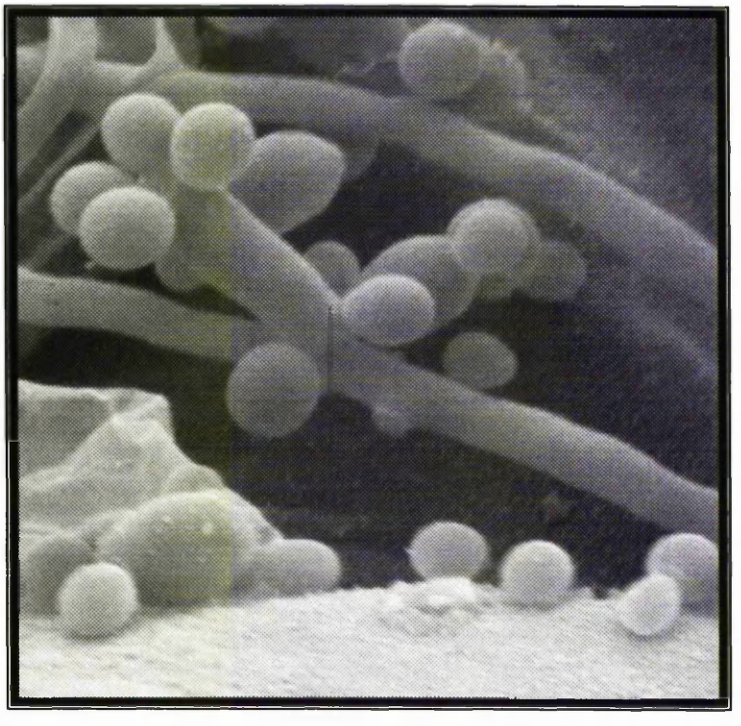

Figure 79: Scanning electron micrograph of *C. albicans* on LSE following 48hr incubation. True hyphal production with clusters of yeast cells (x8000).

Figure 80: Scanning electron micrograph of *C. albicans* on LSE 72hr incubation. Spherical yeast cells with yeast cells, branched pseudohyphae and true hyphae observed. Generalised invasion of the epidermis was detected (x1200).

4.3.3 SEM OBSERVATIONS OF FUNGAL GROWTH ON BRT

4.3.3.1 M. FURFUR ON BRT

As mentioned previously, the dorsal surface of BRT is similar in structure and composition to that of LSE. Similar patterns of growth for both *C. albicans* and *M. furfur* were observed on BRT, with clusters of cells becoming widespread. Budding of *M. furfur* was rarely observed at 24hr (Figure 81 and Figure 82) but large distinct colonies were detected. Budding on BRT became more common after 48hr incubation (Figure 83 and Figure 84). Widespread budding of the majority of cells in the colony was observed but there was no hyphal transformation at that time.

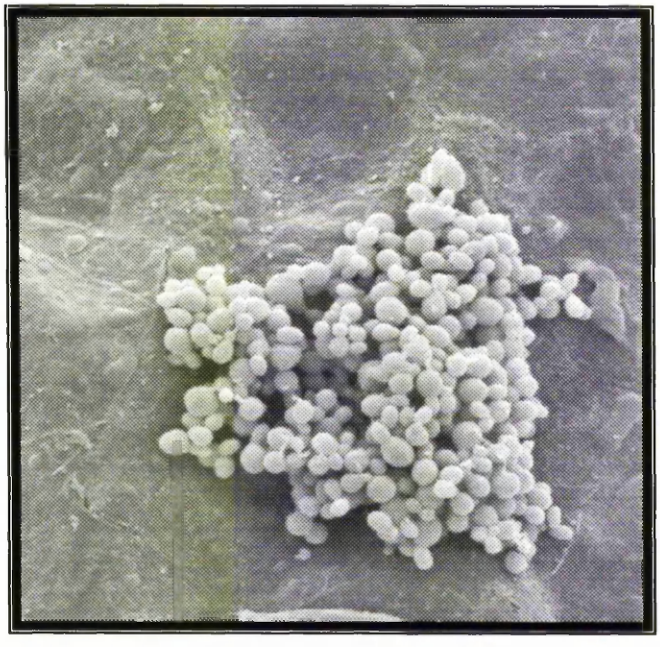

Figure 81: Scanning electron micrograph of *M. furfur* on BRT following 24hr incubation. Ellipsoidal, ovoid and spherical yeast cells observed in clusters with some cells exhibiting unipolar budding (x1200).

Because of the non-homogeneous nature of BRT, different rates and patterns of colonisation were seen in the tissues. The presence of hair follicles on some of the BRT accounted for the accumulation of *M. furfur* on both the hair shaft and follicle, which are known to be a common areas of colonisation for the organism (Figure 84 and Figure 85).

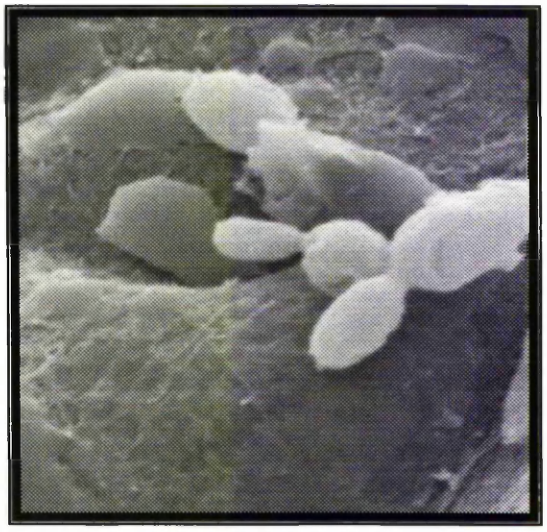

Figure 82: Scanning electron micrograph of *M. furfur* on BRT following 24hr incubation. Budding rarely observed (x3200).

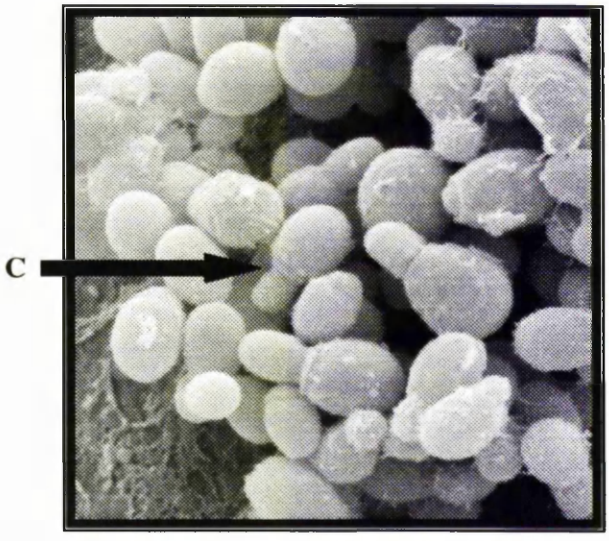

Figure 83: Scanning electron micrograph of *M. furfur* on BRT following 48hr incubation. Extensive budding observed with a distinct collarette (C) (x4000).

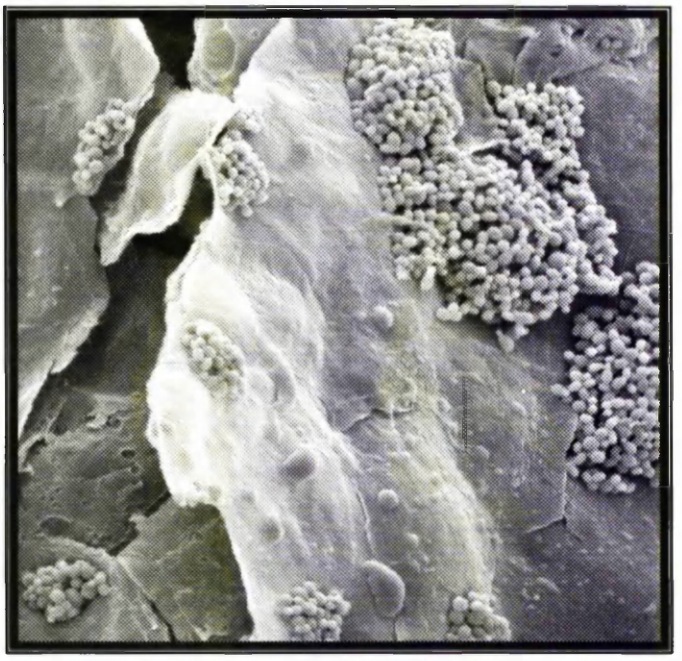

Figure 84: Scanning electron micrograph of *M. furfur* on BRT following 48hr incubation. Clumps of ovoid cells in distinct colonies with budding visible (x1200)

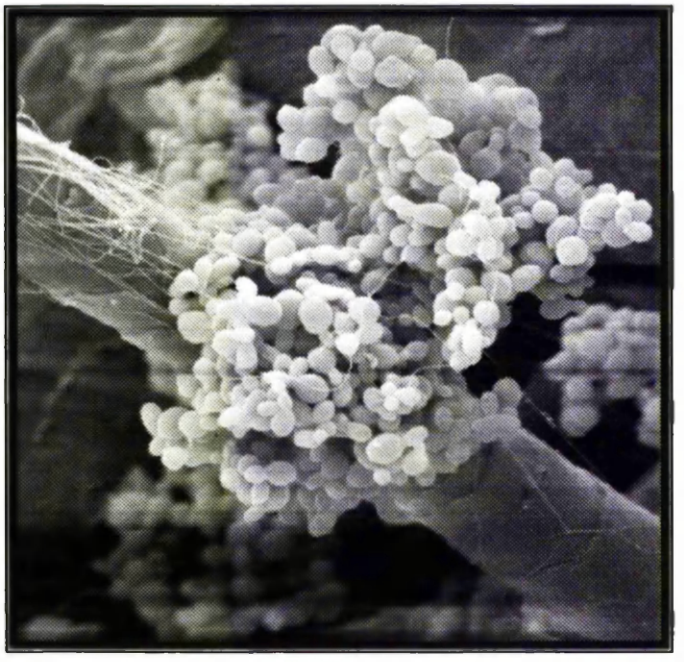

Figure 85: Scanning electron micrograph of *M. furfur* on hair follicle following 48hr incubation on BRT. Accumulation of organism on the hair follicle and exposed shaft with fibrin-like structures connecting yeast-yeast and yeast-hair shaft (x3200).

Growth of *M. furfur* on the hair shaft was associated with fibrous structures that appeared to connect the separate *M. furfur* cells to each other, but also anchored *M. furfur* to the hair shaft (Figure 86). Extensive budding by *M. furfur* was observed on the hair shaft in all donors indicating a positive nutrient environment in this area in all of the donors tested. Budding continued at 72hr incubation and this was associated with discrete clumps of *M. furfur*. Hyphal transformation was not detected in any samples until 72hr incubation (Figure 87) and was still limited at this time.

Figure 86: Scanning electron micrograph of *M. furfur* on a hair follicle after 72hr. Showing a mixed population of spherical and ellipsoidal cells with evidence of budding. Fibrous structures (F) associated with cells (x8000).

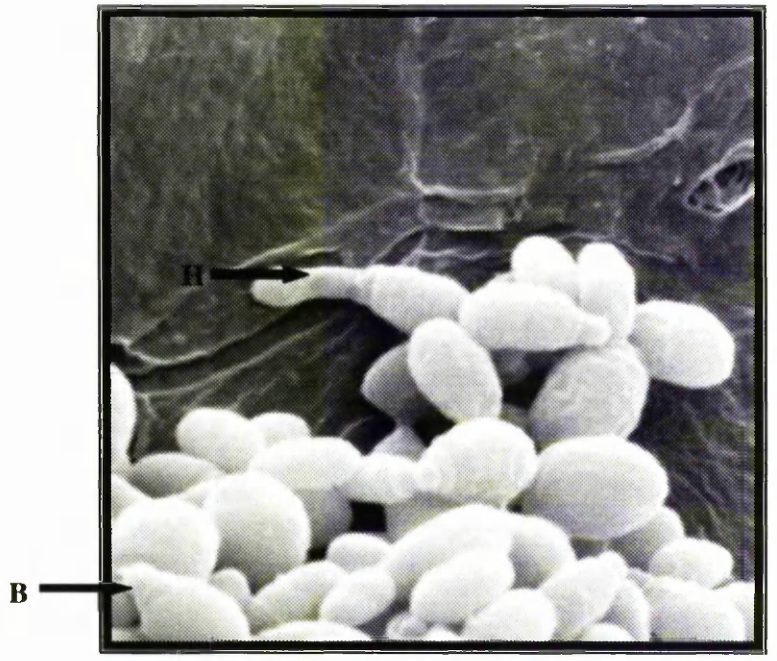

Figure 87: Scanning electron micrograph of *M. furfur* on BRT following 72hr incubation. Clumps of mixed cells with hyphal extension (H) and budding (B) observed (x8000).

4.3.3.2 C. ALBICANS GROWTH ON BRT

Growth of *C. albicans* on BRT was slower than on LSE. *C. albicans* hyphal transformation was however detected on the BRT samples following 48hr incubation. Although it was not as widespread as that observed in LSE, *C. albicans* did show continued production of hyphae from 48hr onwards. Clumps of cells were also frequently observed, often in the skin folds of BRT, with no hyphal extension (Figure 88).

Figure 88: Scanning electron micrograph of *C. albicans* in skin fold of BRT after 48hr incubation (x1200).

Hyphal extension from the perimiter of the *C. albicans* clusters was observed. As Figure 89 shows, distinct zones of colonisation with outreaching hyphae were observed. The transformation of *C. albicans* on BRT occured earlier than the transformation of *M. furfur* on the same time matched donors. Figure 90 demonstrates the presence of *C. albicans* hyhal production on the tissue at 72hr.

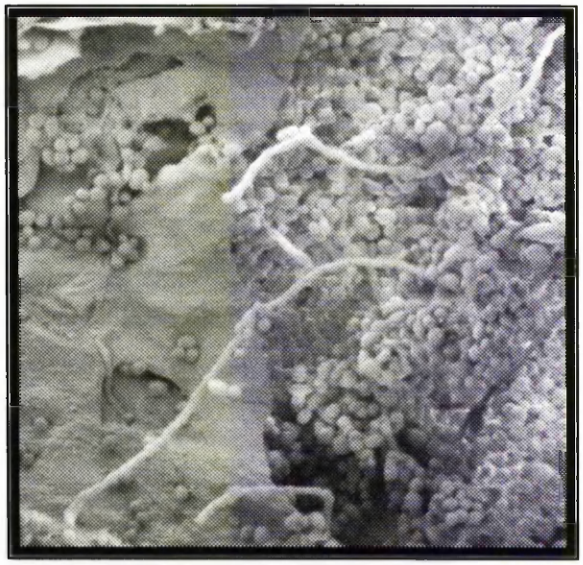

Figure 89: Scanning electron micrograph of *C. albicans* on BRT after 48hr showing clumping of spherical cells with limited hyphal production (x1200).

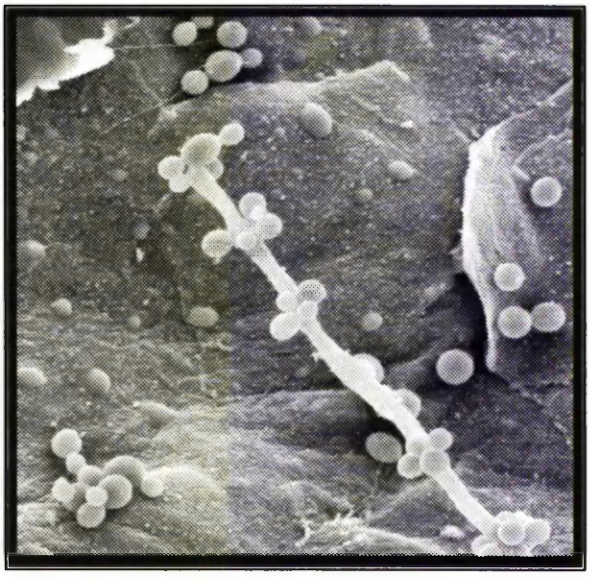

Figure 90: Scanning electron micrograph of *C. albicans* on BRT after 72hr. Single yeast cells, distinct clusters and clusters of yeast cells along the hyphae are apparent (x4000).

4.3.4 HISTOLOGY OF SKIN MODELS

Comparative histology of BRT showed normal epidermal morphology in the excised skin, when examined following H&E staining (Figure 91). After 24hr incubation, the control excised tissue still retained its normal morphology (Figure 92); however, the viability declined with incubation time, as can be seen by the KC vacuolation in the tissue (Figure 93). Following 96hr incubation, control BRT showed a necrotic morphology with large vacuolated cells in mainly the granulosum layer of the epidermis; this was observed using both H&E staining (Figure 93) and PAS staining (Figure 94).

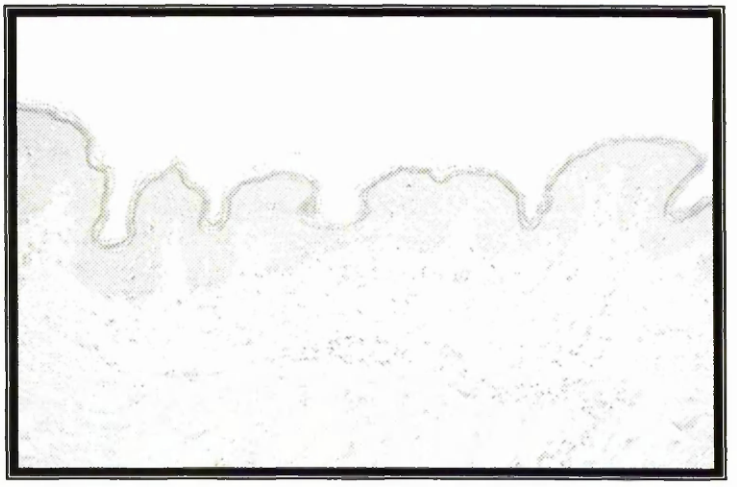

Figure 91: Histological observation of BRT on day of excision (H&E x100).

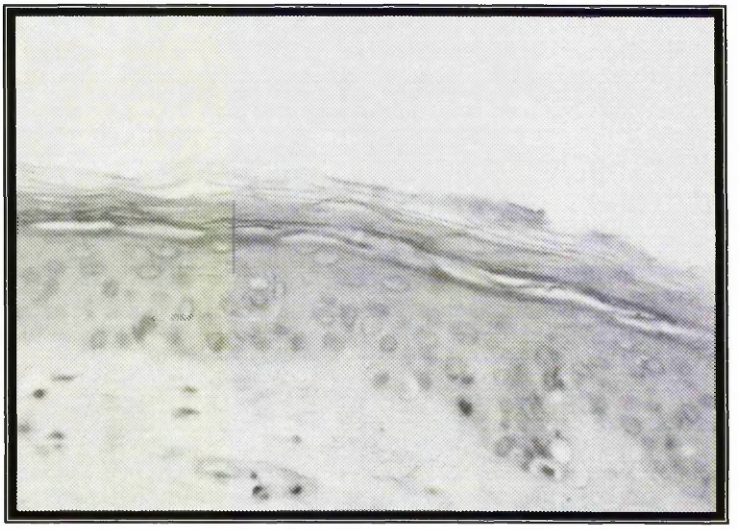

Figure 92: Histological observation of control BRT 24hr post-excision (H&E x400).

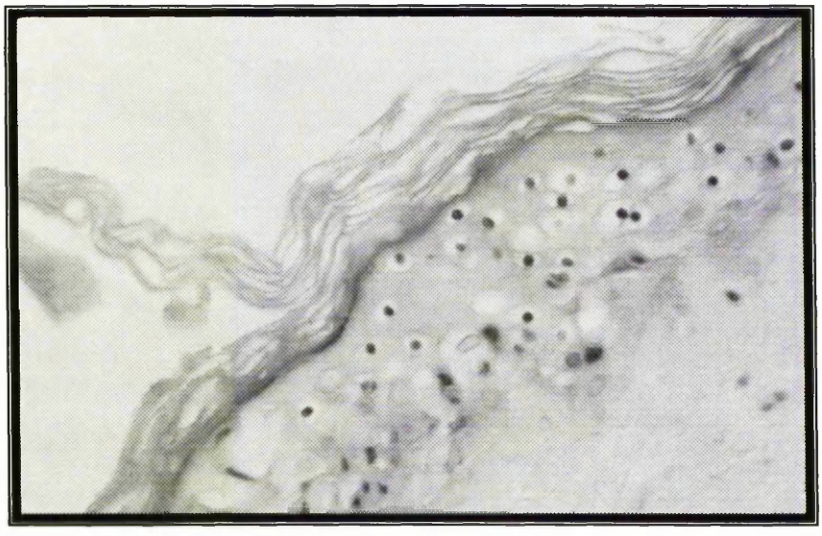

Figure 93: Histological observation of control BRT 96hr post-excision (H&E x400).

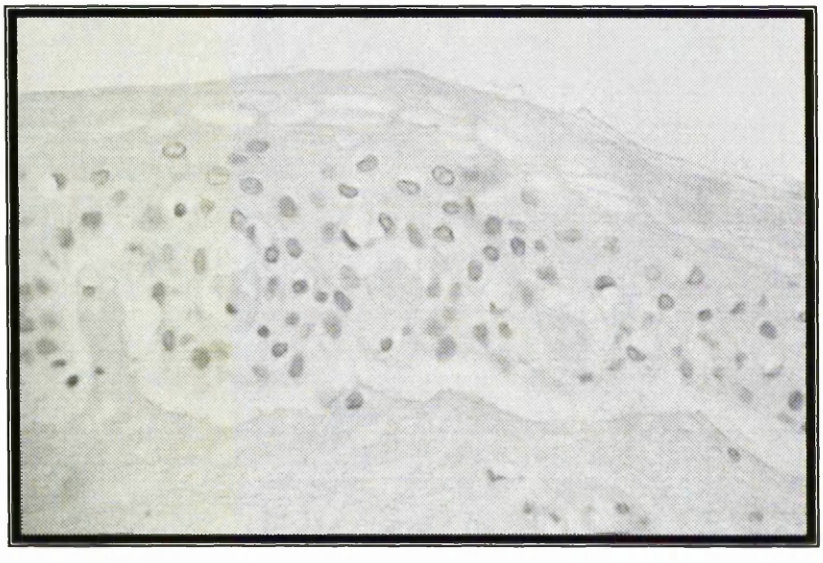

Figure 94: Histological observation of control BRT 96hr post-excision (PAS x400).

The protocol used for formulation of LSEs resulted in viable epidermal-like tissue (Figure 95) that remained viable in excess of 96hr (data not shown). It is important to remember that the LSEs became ready for experiment comencement following 10 days incubation at the air liquid interface. Therefore time zero equalled 10 days incubation at the air liquid interface. The addition of ascorbic acid, as detailed previously, should have improved the lipid content of the tissue, which was not analysed, but hstologically this tissue possessed an *in vitro* like stratification with distinct basal, granular and horny layers (Figure 96).

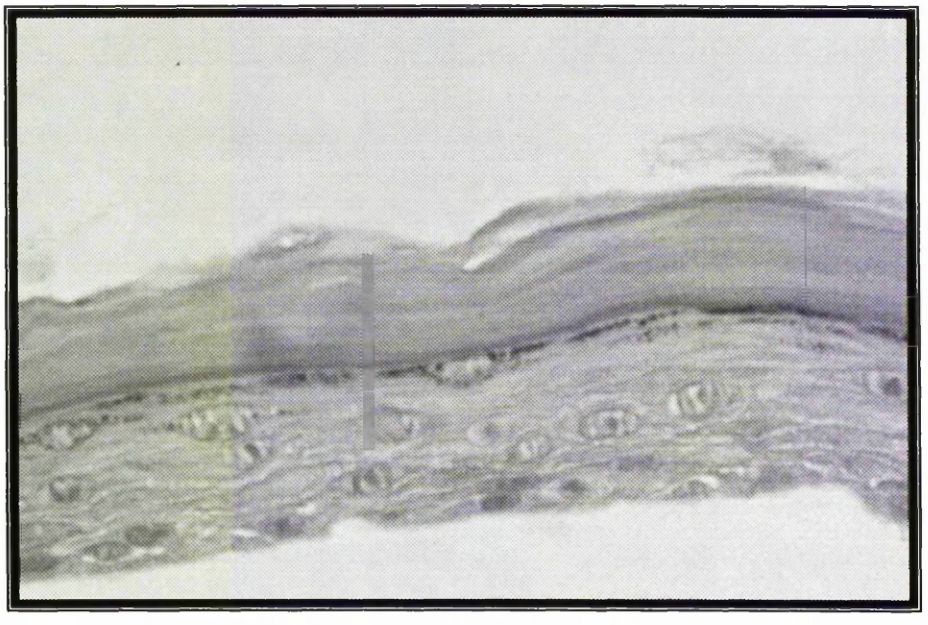

Figure 95: Histological observation of control LSE following 11 days of incubation at air liquid interface; 24hr control (H&E x400).

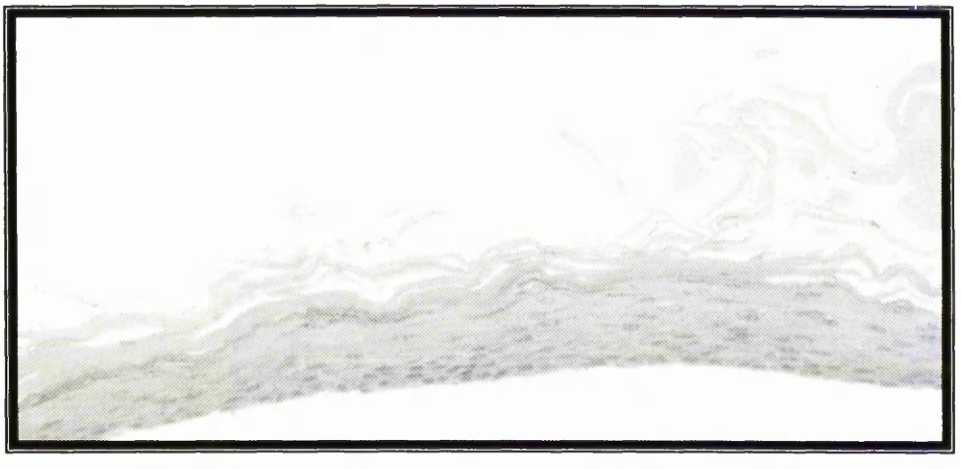

Figure 96: Histological observation of control LSE following 48hr incubation (H&E x250).

Due to the weak nature of the basement membrane/hemi-desmosomal linkage, the epidermal layer of the LSE was poorly anchored to the underlying dermal component and frequently detached during processing (Figure 96). The stratum corneum of the LSE was particularly well developed, due to the hyperproliferative nature of the KCs within the epidermis (Figure 97). In addition, the stratum corneum thickened in response to culture time following incubation at the air/ liquid interface as can be seen by comparing Figure 95, Figure 96 and Figure 97. This was due to increased differentiation of LSE KCs and the absebce of shedding in the humidified protective atmosphere.

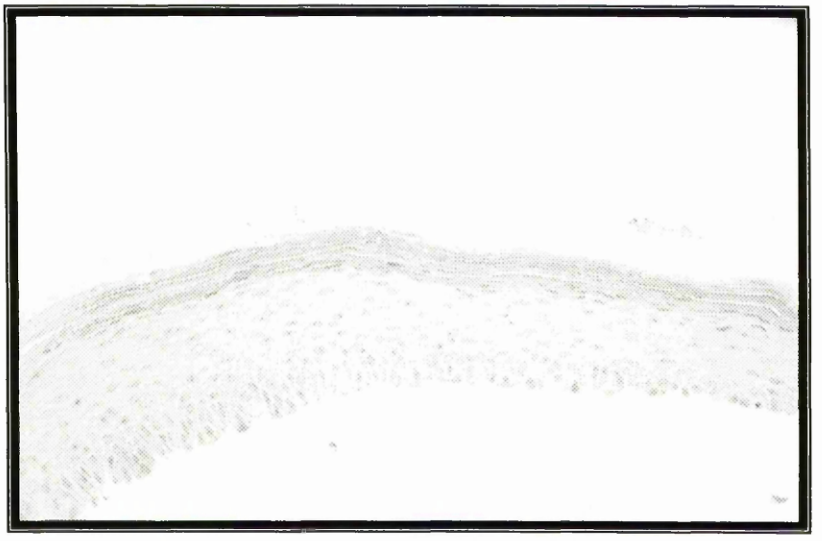

Figure 97: Histological observation of control LSE following 72hr incubation (H&E x250).

4.3.5 HISTOLOGICAL OBSERVATIONS OF FUNGAL GROWTH ON LSE

Growth of both *C. albicans* and *M. furfur* on the skin models appeared to proceed more rapidly on LSE than on BRT. Distinct clumping of *M. furfur* was observed in the stratum corneum of the LSE following 48, 72 and 96hr incubation (Figure 98, Figure 99 and Figure 100). This clumping was in contrast to the hyphal transformation of *C. albicans* in the upper stratum corneum of the LSE at 48hr (Figure 101). Hyphal production by *C. albicans* was restricted to the upper stratum corneum of the LSE and was commonly observed in the tissue at this time. *M. furfur* only began to undergo hyphal transformation following 72hr incubation, with intermittent hyphal elements produced (Figure 102). These cells were, like *C. albicans* at 48hr, confined to the stratum corneum of the LSE but most commonly *M. furfur* can still be seen in untransformed clumps, again in the stratum corneum (Figure 100).

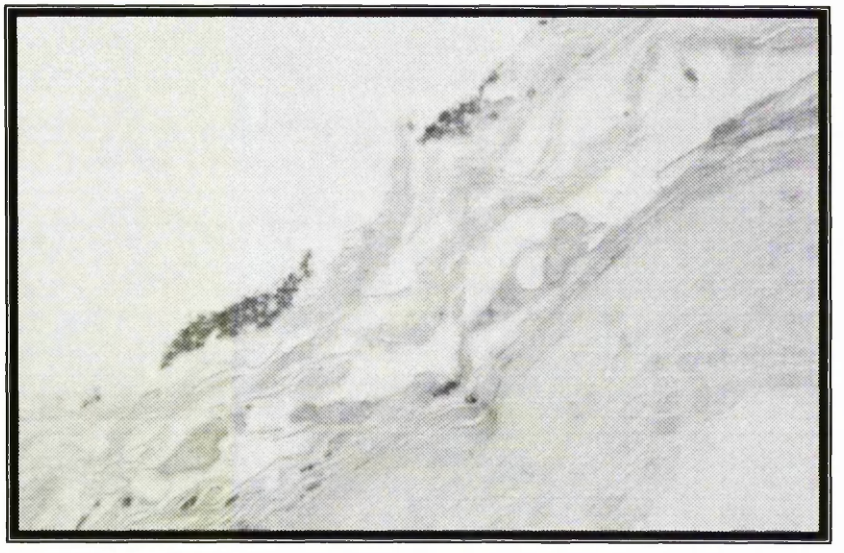

Figure 98: Histological observation of LSE following 48hr co-incubation with *M. furfur*. Clumps of *M. furfur* in the stratum corneum are apparent (H&E x400).

More widespread hyphal production by *C. albicans* could be observed at 96hr (Figure 103). Destruction of tissue by the *C. albicans* hyphae reached the dermis. This destruction was commonly observed, and the remaining LSE tissue was extremely damaged and thus could not be considered viable. If the incubation was continued past 96hr, the stratum corneum of the LSE was not detected by histology. *M. furfur,* however, did not show analogous widespread production of hyphae, and only a few hyphae could be detected in desquamated layers of the stratum corneum of LSE (Figure 102). More widespread clumping of *M. furfur* was observed at 96hr (Figure 100), supporting the theory that the organism does sustain growth on the tissue.

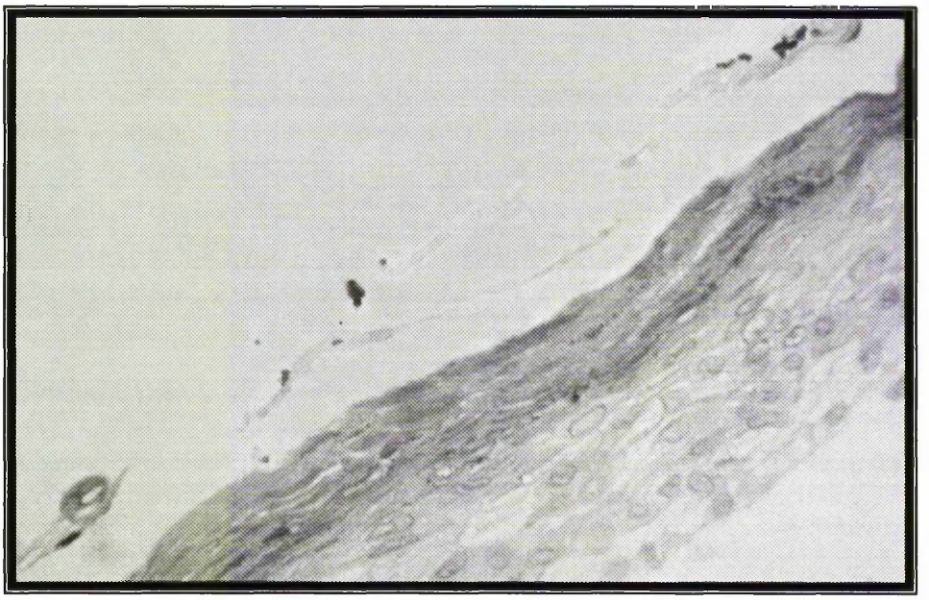

Figure 99: Histological observation of LSE following 72hr co-incubation with *M. furfur*. Yeast budding in the stratum corneum can be seen (H&E x400).

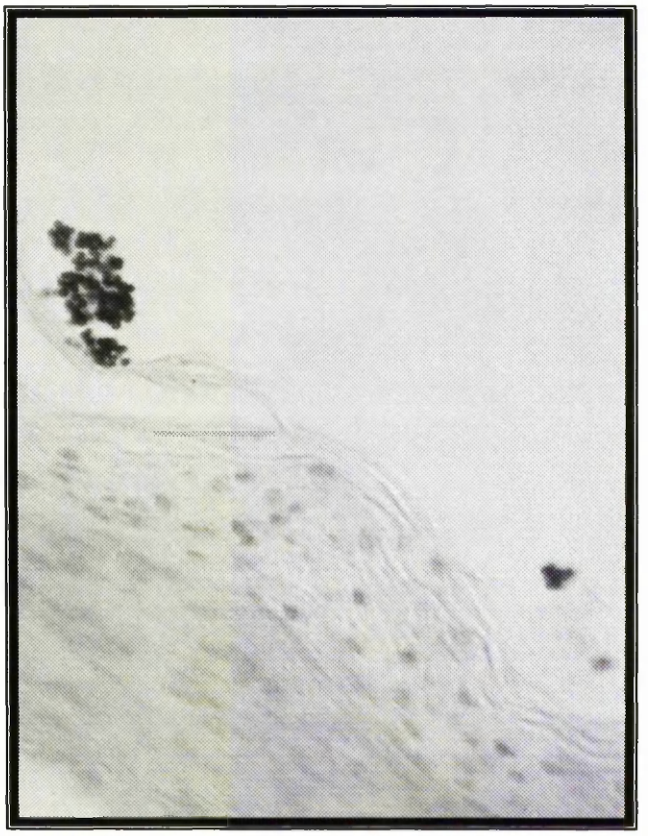

Figure 100: Histological observation of LSE following 96hr co-incubation with *M. furfur*. Clumps of *M. furfur* in the stratum corneum can be seen (H&E x400).

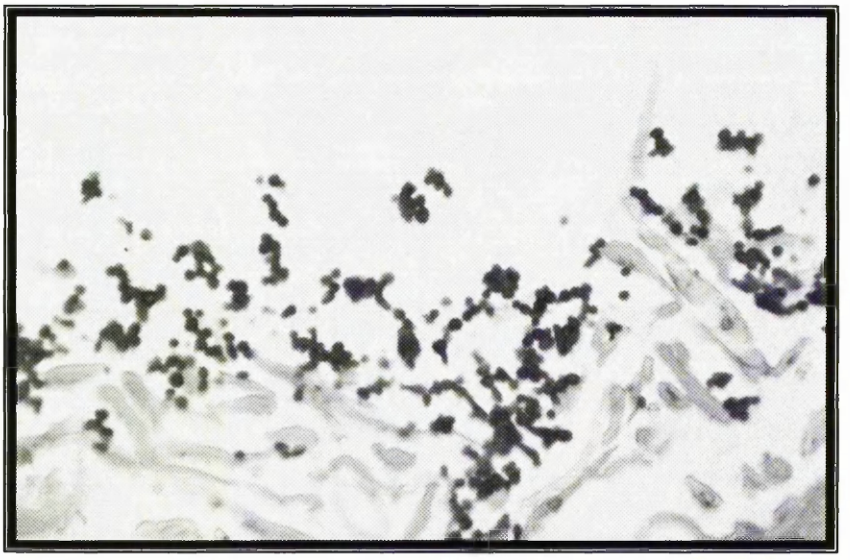

Figure 101: Histological observation of LSE following 48hr co-incubation with *C. albicans*. Clumps of *C. albicans* in the stratum corneum can be seen (PAS x400).

Figure 102: Histological observation of LSE following 72hr co-incubation with *M. furfur*. Clumps of *M. furfur* and hyphal production in the stratum corneum observed (PAS x450).

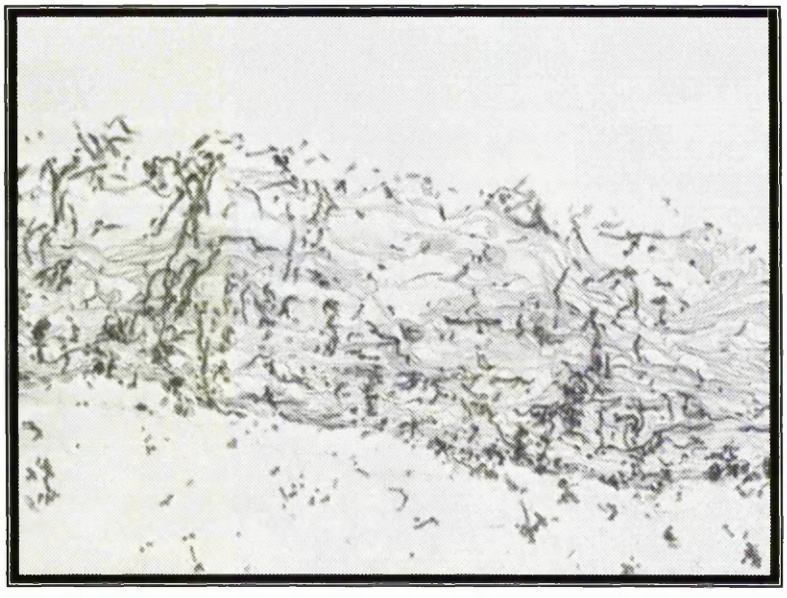

Figure 103: Histological observation of LSE following 96hr co-incubation with *C. albicans*. *C. albicans* hyphae can be seen in the epidermis, spreading down to the LSE dermis (H&E x250).

4.3.6 HISTOLOGICAL OBSERVATIONS OF FUNGAL GROWTH ON BRT

Histological examination of BRT co-incubated with *M. furfur* demonstrated clumping of the organism in the stratum corneum of the excised tissue epidermis following 48hr incubation (Figure 104). Tissue co-incubated with *C. albicans* also exhibited clusters of cells in the upper epidermis, and attachment of *C. albicans* to the desquamated layers of the stratum corneum was widely observed (Figure 105) BRT samples co-incubated with either *M. furfur* or *C. albicans* for 24hr had no organisms in the stratum corneum, and no hyphae were detected. At 72hr clumping of both *M. furfur* (Figure 106) and *C. albicans* became more widespread. In all experiments the organisms were still confined to the stratum corneum of the BRT.

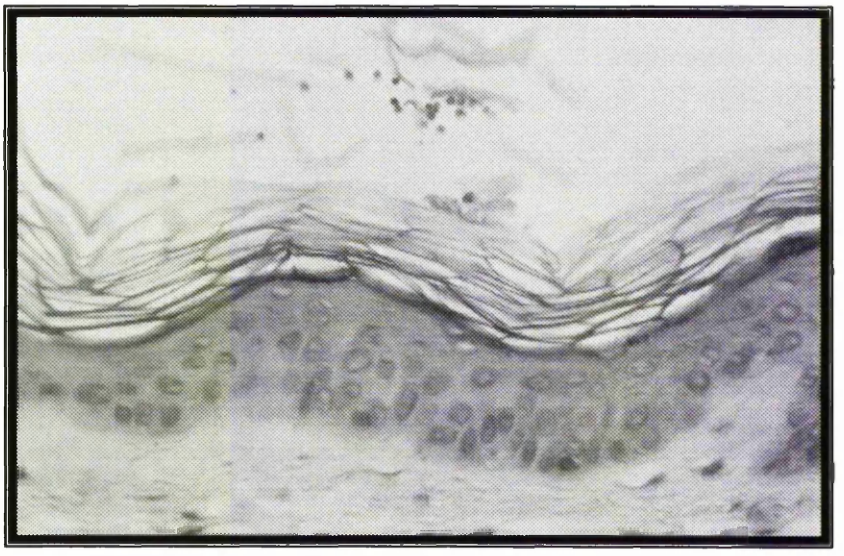

Figure 104: Histological observation of BRT following 48hr co-incubation with *M. furfur*. Clumps of *M. furfur* in the stratum corneum can be seen (PAS x400).

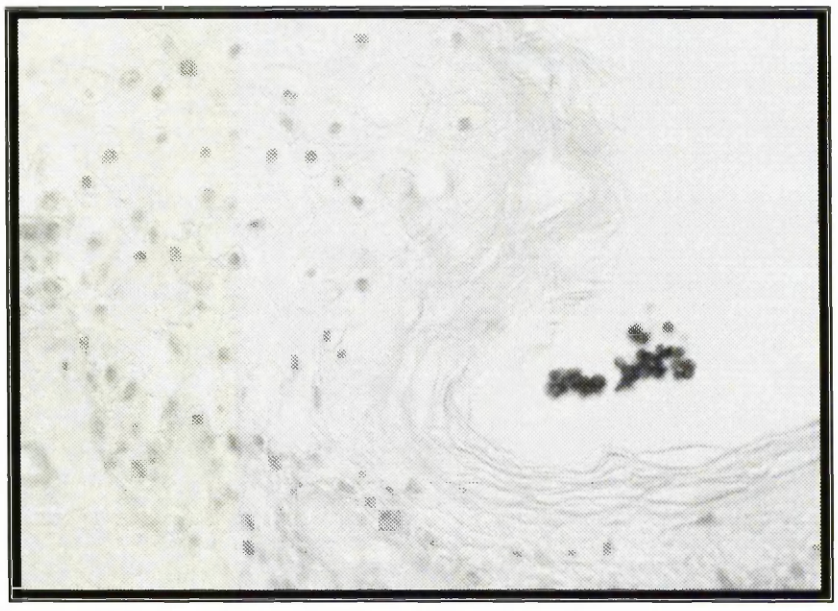

Figure 105: Histological observation of BRT following 48hr co-incubation with *C. albicans*. Clumps of *C. albicans* can be seen (PAS x400).

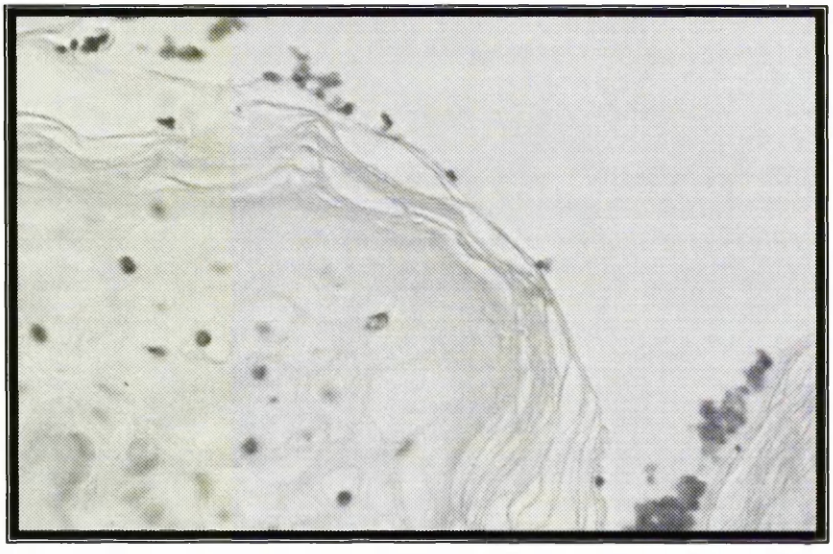

Figure 106: Histological observation of BRT following 72hr co-incubation with *M. furfur*. Clumps of *M. furfur* in the stratum corneum observed (PAS x400).

In some time samples the hyphal production resulted in widespread epidermal damage. Hyphal growth on BRT became extensive at 72hr for *M. furfur* (Figure 107). In general hyphal transformation by *C. albicans* always occurred at 72hr, if not before; however, *M. furfur* did not produce hyphae at all experiments. Tissue from some donors appeared to stimulate little *M. furfur* hyphal transformation (Figure 108), while some donors stimulated extensive hyphae production widespread destruction of the stratum corneum Figure 109). Other inoculated tissue samples showed only limited hyphal transformation of *M. furfur*, and in these cases the transformation was restricted to the upper layers of the stratum corneum (Figure 108). *C. albicans* displayed hyphal transformation repeatedly in tissue incubated for 72hr or less (Figure 110). Extensive destruction of the stratum corneum, as seen in Figure 110, was commonplace with the *C. albicans* hyphae reaching into the dermis of the BRT.

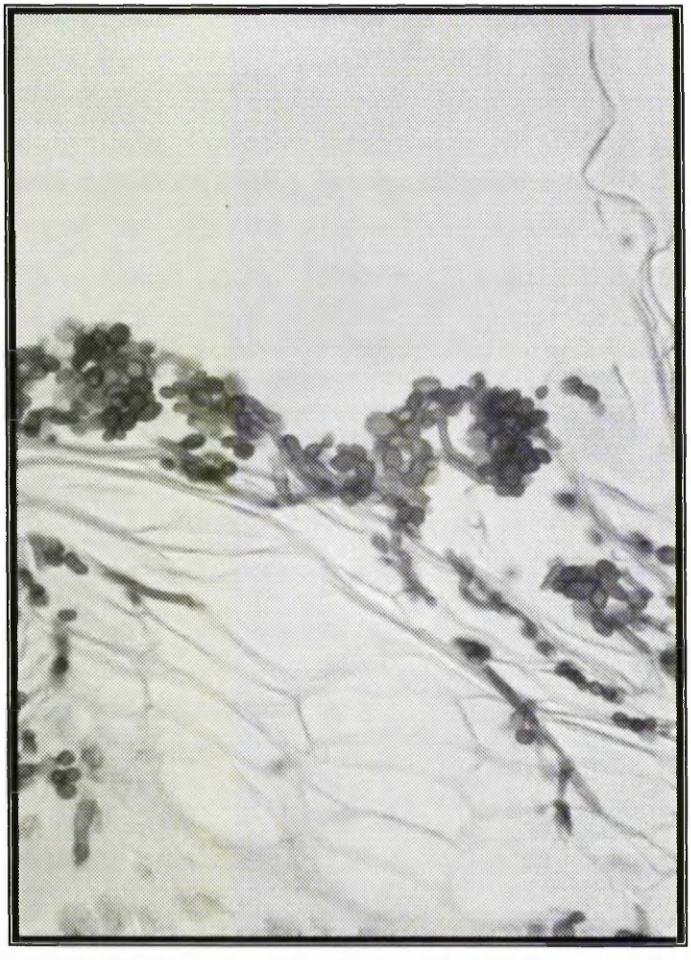

Figure 107: Histological observation of BRT following 72hr co-incubation with *M. furfur*. *M. furfur* hyphae can be seen in the stratum corneum (PAS x450).

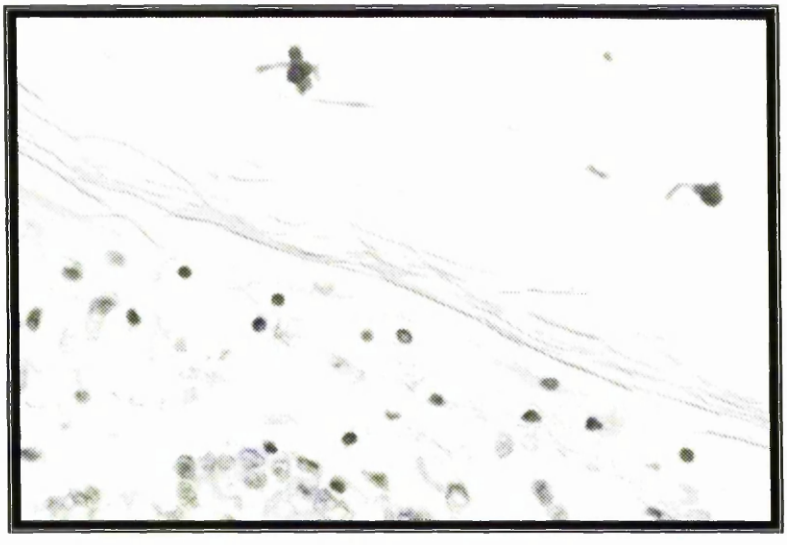

Figure 108: Histological observation of BRT (donor 22) following 96hr co-incubation with *M. furfur*. Hyphal extension into stratum corneum can be seen (PAS x400).

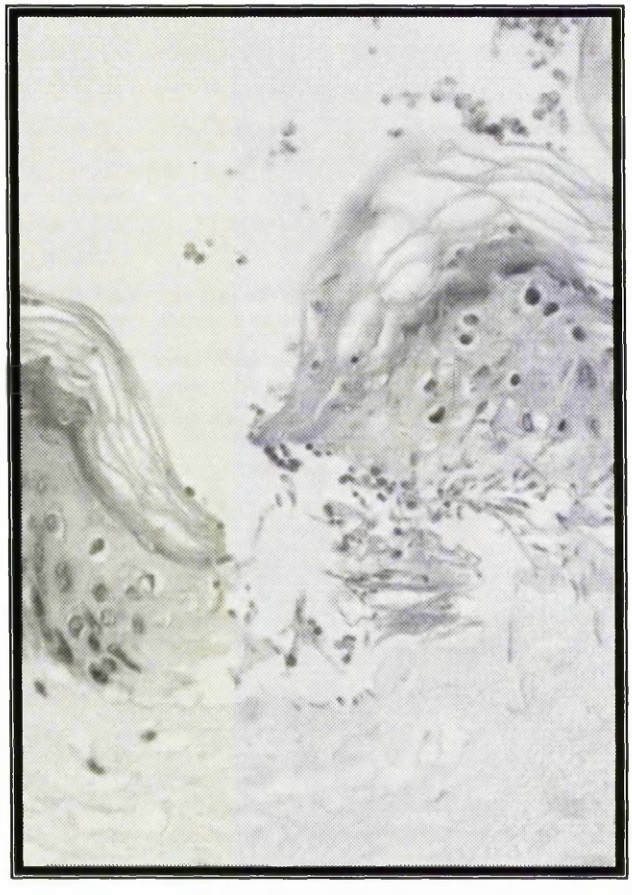

Figure 109: Histological observation of BRT (donor 23) following 96hr co-incubation with *M. furfur*. Destruction of the epidermis by hyphae can be seen (H&E x400).

Figure 110: Histological observation of BRT following 72hr co-incubation with *C. albicans*. Extension of *C. albicans* hyphae into the dermis can be seen (PAS x400).

4.3.7 GROWTH OF FUNGI IN SKIN MODELS

Growth curves for both *C. albicans* and *M. furfur* on the LSE revealed that *C. albicans* had a much higher growth rate on this tissue. Moreover, there was a detectable difference in the growth of both fungi when KCs from different donors were used as a component of the LSE (Figure 111 and Figure 112). The significance of donor differences was highlighted by the very different patterns of growth obtained in BRT from two donors (Figure 113 and Figure 114). While donor 24 appeared to allow a rapid and substantial increase in *C. albicans* cell numbers, there was limited growth on BRT from donor 25. Growth of both was better on LSE than on BRT, as can be seen by comparing Figure 111 & Figure 112 with Figure 113 & Figure 114. The rate and magnitude of growth of *C. albicans* and *M. furfur* was greatly increased on the LSE tissue. This finding is consistent with the histological and SEM data obtained in sections 4.3.5 and 4.3.6.

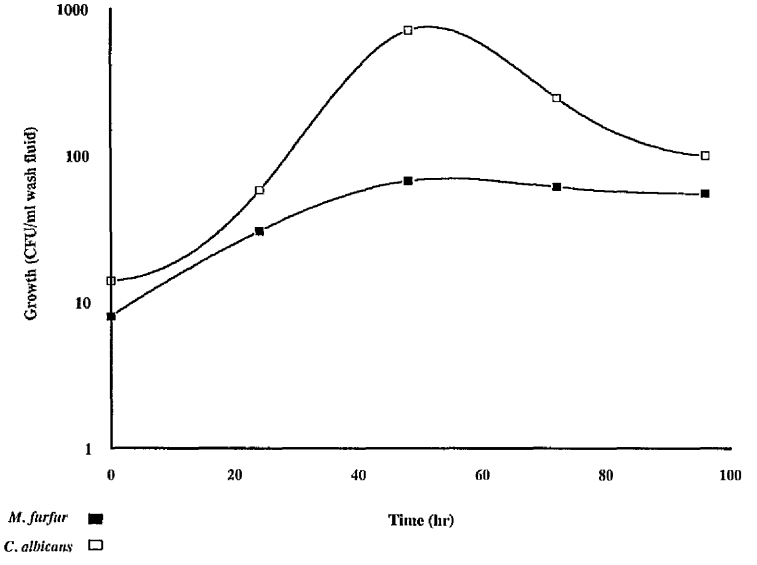

Figure 111: Growth of *M. furfur* and *C. albicans* on LSE, donor 1.

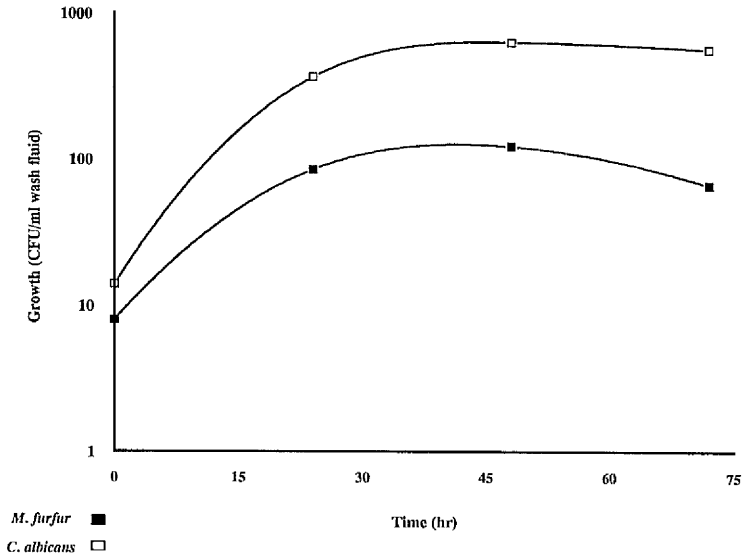

Figure 112: Growth of *M. furfur* and *C. albicans* on LSE, donor 2.

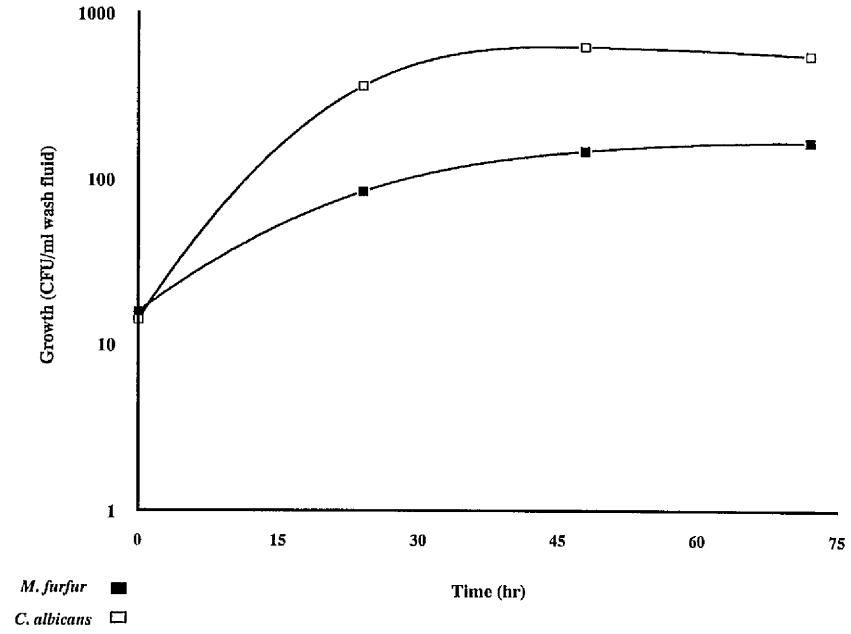

Figure 113: Growth of *M. furfur* and *C. albicans* on BRT, donor 24.

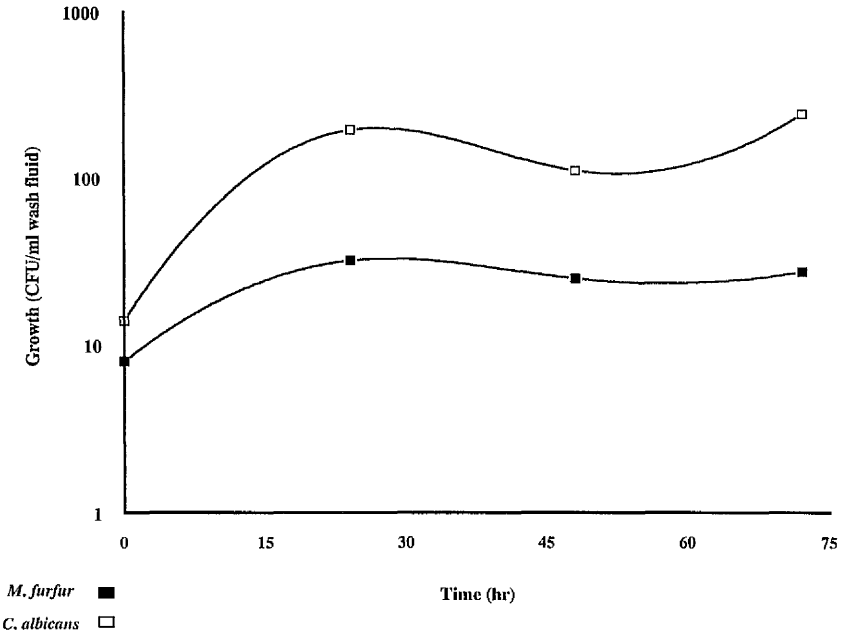

Figure 114: Growth of *M. furfur* and *C. albicans* on BRT, donor 25.

4.3.8 PROLIFERATIVE INDEX OF SKIN MODELS

As can be seen from Figure 115 and Figure 116, there was clear Ki-67 staining of both BRT and LSE. Ki-67 antigen is expressed during active cellular proliferation (G1 stage of cell cycle) thus its expression in a cell indicates active proliferation. Therefore by counting the number of positive cells/1000μm of the basal epidermis, a measure of the proliferation of the tissue could be obtained. Examination of BRT from two donors showed that growth of *M. furfur* and *C. albicans* stimulates an increase in proliferation of the breast tissue epidermis. While *C. albicans* produced a rise in the proliferation of the breast tissue epidermis, *M. furfur* appeared to overtake the *C. albicans* 'stimulation' at 72hr (Figure 117), at a time when the previous data (Figure 113 and Figure 114) indicated growth of *C. albicans* was far greater than that of *M. furfur*. Ki-67 staining was not restricted to the basal epidermis in LSE, but was in BRT. This indicated the increased proliferative index of LSE while in resting phase.

Figure 115: Ki-67 staining of BRT 72hr control

Figure 116: Ki-67 staining of LSE 48hr control.

Due to the rapid growth of *C. albicans* on LSE (Figure 111, Figure 112) it was impossible to obtain a triplicate count of the Ki-67 proliferative index of the LSE in response to *C. albicans* colonisation since the epidermis was often completely destroyed and could not withstand immunohistochemical processing. As Figure 118 demonstrates, *M. furfur* does induce a significant increase in LSE proliferation in response to initial colonisation at 24hr.

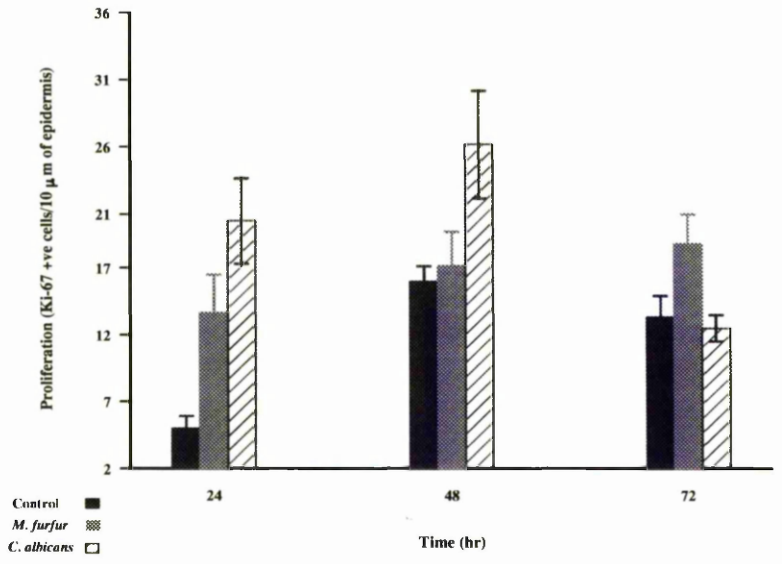

Figure 117: Proliferation of BRT in response to *M. furfur* and *C. albicans* colonisation.

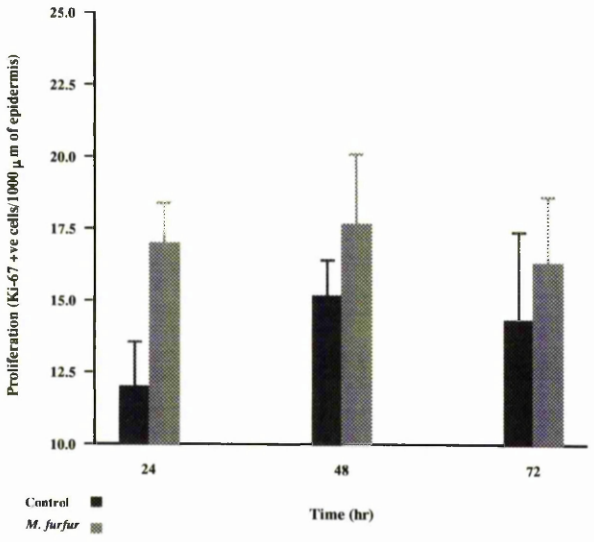

Figure 118: Proliferation of LSE in response to *M. furfur* colonisation.

4.4 DISCUSSION

The colonisation of normal human skin by *M. furfur* has long been recognised and studies of the metabolic requirements of *M. furfur* have established an absolute requirement for an exogenous lipid source. The growth of *M. furfur* has been demonstrated *in vitro*, in animal models (guinea pigs) and in skin models; however, little evidence exists as to the morphology of the organism in model systems. The SEM and quantitative growth measurements carried out in this study attempted to clarify the growth rates and patterns of *M. furfur* and *C. albicans* on BRT and LSEs.

Previous studies have established the role of LSE in investigating fungal infections (Battacharyya et al., 1998). While studies on *M. furfur* infection of LSE revealed the ability of the organism to sustain growth, no evidence was present concerning the differences in donor KCs, and their antimicrobial activity, to inhibit or promote growth. As this study has pointed out, not only do different excised tissues yield different growth patterns of *M. furfur* and *C. albicans*, but the LSE, an apparently homogenous skin model, also affects fungal growth due to the differences in KCs from different donors. As can be seen from both the histological data and SEM observations, fungal morphology varies greatly on different host tissue. While *C. albicans* produced hyphae when grown on BRT from some donors, BRT from other donors sustained only limited growth with little hyphal production. As nutrient stress is often a triggr for hyphal production in *C. albicans* it is possible that the nutrient lacking environment of the LSE did promote hyphal production by the organisms. *M. furfur* also exhibited donor differences in its growth rates and patterns on BRT, with some tissue initiating extreme growth and destruction of the entire epidermis, due to hyphal formation. Other tissue only supported limited cluster-type growth with no hyphal formation observed, however in these models clustering was often widespread over the tissue. It is possible that different strains of organism, had they been used, may have exhibited different colonisation patters however as the strains used were from clinical isolates it is assumed that they would be most likely to act and adhere as pathogens. A putative explanation for differences in LSE growth patterns may be the lipd concentration of the skin equivalent. The LSE, which having been raised to the air/liquid interface 10 days previously never formulates a appropriate barrier function due to its altered lipid profile. The percutaneous penetration of the LSE, which is very high compared to skin, indicates the dysregulation in both the LSEs barrier function and its lipid

profile and it is probable that this irregular lipid concentration greatly effect the lipd sensitive *M.furfur*.

The reverse should be true for BRT where a lipid profile more closely resembling that *in vivo* should exist. This would stimulate growth and differences in host lipid levels in the BRT may contribute to the differences in *C. albicans* and *M. furfur* growth. In addition to this 'donor-difference'; there is also the possibility that the KCs of both LSE and BRT may have varying rates and quantities of HBD-2 production. As demonstrated previously, both BRT, KC and LSE produce different levels of HBD-2 mRNA. Thus, the production of this antimicrobial peptide would directly affect the growth of the organisms on these tissues.

One of the most interesting results obtained in this study is the increase in the proliferative index of KCs measured in these skin models in response to infection. This appears to be the first time that any skin model has been assayed for a direct effect on the proliferation of the epidermal KCs. It would be useful to obtain the proliferative index of biopsied, infected skin, and biopsied normal skin in those individuals suffering from *M. furfur* infections, particularly SD which manifests clinically mainly as epidermal hyperproliferation. As mentioned previously, the rise in IL-1α transcription and secretion in response to *M. furfur*-associated compounds could be responsible for this rise in epidermal proliferation since IL-1α directly acts as a mitogen for KCs. The difference in Ki-67 staining patterns between BRT tissue and the LSE should be noted. As a naturally occurring tissue, BRT exhibits the limited Ki-67 staining of the basal KCs expected of 'normal' skin. LSE, with its hyperproliferative epidermis, reveals Ki-67 staining in the higher layers of the epidermis. This supports the conclusion that LSE is not completely equivalent to *in vivo* skin. However, as long as these limitations are recognised, LSE can still be used successfully in various areas of skin research, and in particular for further studies into *M. furfur* infection.

One of the other points raised in this study highlights the danger of only undertaking histological observation of the growth and extrapolating this to *in vitro* growth. Little growth can often be observed by histological analysis of the tissues while the viability studies (Figure 111, Figure 112, Figure 113 & Figure 114) all indicate that growth occurs on both models, albeit slow in the case of *M. furfur*. Pehaps a better indication of growth and indeed the patterns of growth obtained, comes from studying the SEM data. SEM allows an overall view of the dorsal surface of both tissues, allowing the examination of the

patterns and extent of both *M. furfur* and *C. albicans* growth on these tissues. I believe SEM therefore allows a more detailed examination of the nature of the organisms growth on these tissue and thus I believe it should be used in preference to histology in such studies.

Hence what is the validity of using these skin models as directly comparable to *M. furfur* infection *in vivo*? Firstly to employ these models it is essential to use several donors, both for the formation of the LSE (KC donors) and for BRT (use a wide range of tissue donors). By measuring the ability of *M. furfur* and *C. albicans* to grow on various donor-derived tissues, a pattern of *M. furfur* and *C. albicans* growth may be revealed which is dependent upon site of isolation of tissue or KCs. The lipid content of the skin is determined by KC and sebum production from the sebaceous glands, therefore it is possible that the BRT in particular would have large variations in the lipid content of the epiderms as any sebaceous glands are still intact in the tissue.

5 GENERAL DISCUSSION

The question around which most *M. furfur* research, and indeed this thesis is based upon, relates to how *M. furfur* initiates cutaneous infections. While remaining a commensal for the majority of the population, *M. furfur* is known to cause a myriad of dermatological disorders that have a range of manifestations. It is generally accepted that commensals remain commensal because of their weakly pathogenic nature and the effectiveness of host defences in maintaining a limited population. If this is true, two hypotheses present themselves as the most logical explanation for the establishment and progression of these diseases. Either some component of the organism is altered, resulting in its increased pathogenicity, or some aspect of the host environment is altered, initiating the pathology of disease. Several investigators have attempted to address this question by studying colonisation patterns in *M. furfur*-associated disease and the differences in host response between infected and non-infected individuals[41]. The results obtained are inconclusive, for reasons that will be discussed. However it seems likely that differences in host reactions predominate in the transition from *M. furfur* colonisation to *M. furfur* infection.

5.1 ORGANISM FACTORS

Firstly focusing on the organism, it is reasonable to assume that the reversion from commensal to pathogen in *M. furfur*-associated disease could be due to differences in the organism. Many pathogenic organisms possess virulence characteristics that are in addition to their species classification (eg. *E.coli* 0157). Despite the innocuous nature of *M. furfur*, it is possible that certain strains of the organism may contain pathogen-associated factors that contribute to its infectivity.

5.1.1 TYPING OF *M. FURFUR*

Typing of *M. furfur* on skin indicates the presence of three serovars that specifically react with anti-*M. furfur* antiserum, and hence are able to elicit an immune response. While no differences in colonisation rates between these serovars exist in PV and SD infection, the

[41] For the purpose of this discussion, infected individuals are those suffering from PV, SD folliculitis etc, while non-infected individuals are those who are colonised but do not suffer from any recognisable disease.

serovar types can be isolated from distinct areas of the body. Some immunological differences in the host reaction to these organisms can be identified in infected individuals where serovar A stimulates a significant increase in IgG and a decrease in IgA in SD and PV respectively (Ashbee et al., 1994). The difference in the immunological reaction by infected individuals indicates that a difference in host response to the organism is responsible for the transition to infection since no difference in organism density or overall numbers could be detected. Thus, induction of the immune response as described in this study, is an organism-independent phenomenon, based on the fact that uninfected controls had the same type and quantity of organisms as infected individuals.

5.1.2 *M. FURFUR* CLASSIFICATION

One of the main problems in identifying pathogenic *M. furfur* strains has been the difficulty in classifying the *Malassezia* species and the problems with nomenclature. Until recently the identification of *Malassezia* species has relied upon classical microbiological, morphological and metabolic identification mechanisms which are methods open to errors due to the similar characteristics of all the organisms in the *Malassezia* species [42]. Using microscopy and biochemical analysis, a mixed population of *M. furfur, M. globosa* and *M. sympodialis* has been identified on normal human skin (Aspiroz et al., 1999). This is contrary to the historical evidence that only *M. furfur,* while under the nomenclature *Pityrosporum orbiculare,* colonises human skin with an increase in organism density on the chest and back (Faergemann et al., 1983c). The identification of a mixed population on human skin has also indicated that *M. furfur* predominates in both PV and SD while *M. sympodialis* is mostly isolated from normal skin (Makimura et al., 2000). Perhaps this is the problem in clarifying the pathogenicity of *M. furfur:* that *M. sympodialis* is isolated as a commensal on normal skin while *M. furfur* is the pathogenic *Malassezia* species. If this is the case, then a study of the differences in metabolism of these organisms would be appropriate, to determine the pathogenic factors which may initiate disease. One study has compared the growth of *M. furfur* and *M. sympodialis,* indicating very different metabolic pathways and patterns whereby *M. sympodialis* produces significant ammount of free fatty acids and ethyl esters, while *M. furfur* produces only small amounts of both metabolites (Mayser et al., 1998a). The contribution of these metabolites to the initiation of infection

[42] *M. furfur* alone can exist in spherical, globose and hyphal forms.

has never been established, as they have not been tested on any kind of skin model; hence, the exact relevance of the differences in metabolism between these two organisms cannot be clarified. The confusion surrounding both nomenclature and classification of *Malassezia* species must be resolved in order to establish *M. furfur* as the human *Malassezia* pathogen. A retrospective study, utilising the novel molecular typing methods (Makimura et al., 2000) needs to be carried out on a large dataset of clinically isolated *M. furfur*. In addition, the metabolic profiles of both *M. furfur* and *M. sympodialis* would have to be analysed, and different metabolites tested on skin models, to determine whether a pathogenic strain of *M. furfur* exists.

5.1.3 FUNGAL COLONISATION

Putting aside the possibility that *M. furfur* is the primary pathogen of *Malassezia* species, there are several other *M. furfur*-associated factors that may contribute to disease. One of the most obvious possibilities is that increased carriage of the organism, whatever *Malassezia* species it might be, would result in an organism overload whereby the host defence is overwhelmed, resulting in disease. *M. furfur* colonisation begins in infancy with the organism detected during the first 12 weeks of life. In infantile seborrheic dermatitis, the infant equivalent of SD, *M. furfur* is cultured from 90% of infected children, compared with 20% of the non-infected population (Broberg and Faergemann, 1989). This study directly conflicts with more recent data obtained on ISD and *M. furfur* colonisation (Broberg, 1995) and indeed it is the only study which supports a link between increased colonisation with *M. furfur*-associated disease in the normal (non-HIV positive) population. The rate of *M. furfur* colonisation does increase with age, peaking at puberty, directly relating to the peak in sebum production by the host. Hence the rise in colonisation rates is not due to an increase in the organism's pathogenicity, or the growth of a pathogenic strain of *Malassezia*; rather, this is a host-derived alteration of the habitat of the resident *M. furfur* population. *M. furfur* colonisation is greater in the Caucasian population, compared to the black population (Noble and Midgley, 1978), and if *M. furfur* load was responsible for infection a correlation with increased colonisation and increased infection might be expected in these populations. No detectable difference in infections between the races exists in the epidemiological data, thus supporting the theory that fungal density does not increase pathogenicity.

Direct examination of SD and non-infected individuals yields conflicting results on whether *M. furfur* carriage is increased in infection. Bergbrant and Faergemann (1990) investigated quantitative differences in *M. furfur* carriage between SD and non-infected individuals, and reported that there was no significant difference in numbers of organisms between the two groups (Bergbrant and Faergemann, 1990). This finding contrasts greatly with the data of Heng et al (1990) which revealed a relationship between *M. furfur* carriage and SD severity. The variation in results may be due to differences in the quantitative methods used by these two groups. Some studies which examined the HIV-positive population, support the conclusions of Heng et al. For example a greater density of *M. furfur* carriage could be detected on HIV-positive individuals, without *M. furfur* disease, than non-HIV positive individuals (Pechere et al., 1995). A weak trend between organism load and increased severity of SD in the HIV positive population has been identified by two groups (Ross et al., 1994; Schechtman et al., 1995b). Thus there appears to be a relationship between increased density of *M. furfur* in the non-infected HIV-positive population and limited evidence of an increase in SD severity with increased *M. furfur* colonisation in this populationA possible explanation for this is that in the HIV-positive individual the normal immunosurveillance, which protects the skin and all other lymphoid-associated tissues, is depleted and unable to keep in check the proliferation of its commensals. This is supported by data relating to the colonisation of the mucosa in HIV-positive individuals, with organisms previously considered as commensals such as *Candida* species reverting to pathogenicity. Regarding PV infection, only one study by Faergemann (1984) has suggested the possibility of a slight correlation between PV and *M. furfur* colonisation. However, recent studies indicate that no difference in either the serotype or density of *Malassezia* species exists in PV (Ashbee et al., 1993).

Differences in the ability of the organism to adhere to the stratum corneum would account for any increased colonisation. However, no differences in the ability of *M. furfur* to bind to epithelial cells from different areas or differences using *Malassezia* species has been detected (Faergemann et al., 1983a). In addition, no difference in the ability of organisms isolated from SD to bind to stratum corneum cells has been reported (Schechtman et al., 1995a). All of these reults are consistent with the theory that an altered host response to the organism is responsible for infection. As no conclusive evidence exists for a difference in the rate of colonisation between infected and non-infected individuals, the data regarding

HIV infection also support the theory that host factors are responsible for reversion to infection and this is further strengthened by the situation observed in AD. AD presents on the skin as a chronic eczematous eruption which often exhibits elevated *Staphylococcus aureus* infections and elevated IgE serum levels. While there is major alteration of the host skin of an AD sufferer, there is also a significant increase in *M. furfur* colonisation in patients with AD (Hiruma et al., 1999). However the difference however in colonisation in AD, like that observed in HIV, should be considered a difference in the host as opposed to the organism, and as such it will be discussed later.

5.1.4 METABOLITE PRODUCTION

One final organism-related factor which could affect its pathogenicity is fungal metabolism and specifically whether clinical isolates of *M. furfur* have metabolic properties different to that of non-pathogenic strains. While many metabolic products and putative by-products, like dicarboxylic acid and lipase, would account for some of the pathology in cutaneous *M. furfur* infections, no study directly comparing clinically isolated *M. furfur* and laboratory subcultured *M. furfur* has been carried out.

Previous studies have indicated that the antigenicity of *M. furfur* changes according to culture time and growth phase of the organism. Antigenic components isolated from *M. furfur* varied with culture time in their ability to bind IgE in an ELISA, with a rapid decline in antigenicity detected at 4 days (Zargari et al., 1995). Declining antigenicity may be due to the decreased protein concentration in these extracts. It is possible that a nutrient rich environment would encourage the establishment of an exponential population on the skin, resulting in the production of more antigen and thus initiation of disease. In contrast a slow-growing *M. furfur* that would be maintained as a transient population would remain largely inert due to its low production of antigen. This theory is supported by the difference in rates of growth observed in *M. furfur* and *M. sympodialis* (Mayser et al., 1998a), however, no definitive studies have been performed to determine if this is the case in infection.

5.2 HOST-DEPENDENT PATHOLOGY

Based on the currently available data, little difference in the distribution, metabolism or

density of *M. furfur* can be identified; thus the hypothesis that disease is in fact an abnormal host response to this normal commensal prevails. To determine what host factors are responsible for disease pathology, it is necessary to examine differences in the host environment and host immune response which may alter the antigenicity of the organism.

5.2.1 LIPID DIFFERENCES

Individual differences in host sebum levels would be the most obvious variation which could affect *M. furfur* infection. As an obligatory lipophile *M. furfur* has a fastidious requirement for long chain fatty acids, as is indicated by its predilection for the sebaceous areas of the body (Porro et al., 1976). It is reasonable to assume that an optimum host lipid profile may be present which, while not increasing density of the organism, may increase the pathogenicity of the organism by switching its metabolism in some way. Studies on PV and SD-positive individuals indicate a higher lipid content of the skin of infected individuals, as compared to controls (Bergbrant and Faergemann, 1989). In addition, treatment which reduces the levels of lipid secretion initiates resolution of SD in some individuals (Bergbrant, 1995). As declining lipid levels correlate to declining *M. furfur* colonisation a concomitant rise in *M. furfur* levels in infected individuals might be expected but as discussed previously, only tenuous data link infection with high *M. furfur* numbers. Because of the postulated differences in lipid metabolism between *M. furfur* and *M. sympodialis,* the altered lipid profile of a host might encourage growth of one species over the other, eliciting disease. Despite the possibility that the lipid profile does contribute to the pathology of infection, the question remains as to how *M. furfur* initiates the symptoms associated with disease. For reasons that will be discussed it seems that the host-immune response, or lack of it, is the primary host-factor responsible for *M. furfur* induction of infection.

5.2.2 GENERAL HUMORAL IMMUNITY

To establish if the host immune response is integral to *M. furfur* infections, it is necessary to compare the immune response of infected and non-infected individuals to elucidate possible pathways of activation. Much of the recent published research has invstigated the humoral immune response of the host to *M. furfur*. Firstly anti-*M. furfur* antibodies become detectable in the population at approximately 11years of age (Faggi et al., 1998), with a maximum immunoprecipitation formed at age 40. Hence, while the organism can be

detected and cultured from subjects under 11years old, it is not until the onset of puberty that the organism manages to elicit a detectable immune response. It is of course possible that the increase in sebum concentration of the skin at puberty stimulates the putative *M. furfur* and *M. sympodialis* growth axis, allowing the stimulation of the immune response. While an age-dependent increase in the *Malassezia*-specific immune response is observed, this does not explain the initiation of disease by *M. furfur*.

5.2.3 HOST IMMUNE RESPONSE IN SD

In SD there is a myriad of conflicting data regarding the cellular and humoral immune response of the diseased host to the organism. One of the first studies demonstrated that there was no difference in *M. furfur*-specific IgG levels between healthy controls and SD infected individuals (Bergbrant and Faergemann, 1989). Using a different protein component of *M. furfur* this same group later measured a significant decrease in anti-*M. furfur* IgG in SD sufferers (Bergbrant et al., 1991a). Because of the selection of a different antigenic component these two studies cannot be directly compared and this problem prevails throughout all the *M. furfur* infection/ immune reaction studies which have been published. As a standard antigen has not been used throughout, and because the protein component is thought to be more antigenic, these results may indicate an immunosuppressive effect, initiated by *M. furfur*. Despite the lack of standardization it is fair to point out that this same group detected significantly increased IgG levels in SD (Bergbrant et al., 1991b). In this study T lymphocytes from SD sufferers exhibited subnormal stimulation by *M. furfur* antigen, with overall higher levels of circulating T-cells and decreased peripheral blood mononuclear cell proliferation in response to *M. furfur* detected in the SD patients. These studies combined would indicate that an altered cellular response to *M. furfur* is responsible for infection but this theory has recently been challenged by Bergebrant et al (1999) who, on testing the lymphocyte transformation response of SD patients, could not find any difference between controls and patients. In addition, the previous studies by Bergebrant et al, which suggested the significance of the humoral immune response in SD, have recently been refuted by Parry and Sharpe (1998). They demonstrated that there was no detectable difference in the IgA, IgG and IgM *M. furfur*-specific response between controls and SD, so whether SD is a result of a T-cell immune dysregulation or an altered humoral immune response is still unclear.

Evidence from the HIV+ population does distinctly point towards a T-cell dependent dysregulation in SD and this is the most powerful evidence available regarding the host reaction in *M. furfur* infections. The main problem with these studies is the non-standardisation of antigen components and this situation should be resolved by the recent cloning, characterization and expression of a group of *M. furfur* antigens, Malf 1-10. I the present study, *M. furfur* cell wall, whole cells and cytoplasmic extracts were used, and these to were obviously not standardised antigen preparations. The Malf 1 antigen is thought to be cell wall-associated. Thus allthough purified Malf components were not available these antigens used were probably adequate to gauge the stimulatory capacity of *M. furfur* for the innate immune response of the skin models. However, it would be interesting to utilise these characterized antigens in any future experiments. Such studies should allow a more definitive conclusion to be drawn.

5.2.4 HOST IMMUNE RESPONSE IN PV

While PV displays similarities in its pathogenesis to SD, with mild hyperproliferation of the epidermis, the distinct pigmentory changes in PV indicate a unique pathology. Recent studies have demonstrated the capacity of *M. furfur* to significantly decrease the lymphocyte transformation response of patients with PV and some differences in this response were detected using different antigen types. For exampl increased reactivity of PV lymphocytes to *M. furfur* serovar B as compared with ither serovars, has been reported (Ashbee et al., 1994). This substantiates and supports the oval exoantigen study of Silva et al. (1997) which suggests that there is no difference in the host immune response in PV, just a stimulation of the normal immune response due to a switching of the dominant species in the mixed colonizing *Malassezia* population.

Histological observations in patients with PV illustrate dilated blood vessels with moderate B and T-cell infiltration. However, few studies have investigated the significance of the cellular immune response in PV, allthough some evidence points towards a dysregulated T-cell response. A study of changes melanin synthesis might help to clarify the situation. Because melanin synthesis is particularly sensitive to trauma, and the resulting cytokine stimulation, this would be a logical candidate for an investigation of the origin of altered immune response in PV.

5.2.5 ATOPIC DERMATITIS

While AD is a disease not caused by *M. furfur* the organism has been shown to exacerbate the condition in certain individuals. Hence the atopic individual is a prime example of how a pre-altered host immune response is partially responsible for the ability of *M. furfur* to elicit disease. By examining the immune response of AD patients to *M. furfur*, a potential mechanism for *M. furfur* activation or evasion of the 'normal hosts' immune response may be revealed. This is exactly what several investigators have attempted by examining the different cellular and humoral responses to *M. furfur* in the AD patient, as compared to controls. AD is a multi factorial disease which presents as inflammation of the respiratory tract, epithelia and skin when they come into contact with allergens. Overall increased IgE responses to a range of environmental allergens result in disrupted respiratory tract and skin functioning, with hypersenstitivity to these aeroallergens and contact allergens. In the skin an underlying skin abnormality is thought to be responsible for the delayed type contact hypersensitivity mounted in response to allergen contact. Th2 cells and IgE accumulate at the site of allergen contact, with IgE stimulating mast cell histamine release and the stimulation of the cytokine cascade which follows, manifests as red erythematous skin often with underlying puritis. While aeroallergens like the dust mites are known to exacerbate the atopic rhinitis condition, skin commensals have only recently been considered to exacerbate inflammation in AD. The first major study implicating *M. furfur* in AD was carried out in 1985 (Waersted and Hjorth, 1985). It was shown that ketoconazole treatment of individuals with head and neck dermatitis resulted in disease resolution with declining severity of the inflamed areas. Hence a role for *M. furfur* in stimulating AD was hypothesized. This was further supported by work which demonstrated that 80% of AD sufferers had a positive skin reaction to *M. furfur* (Jensen-Jarolim et al., 1992) and more recently it has been shown that the addition of *M. furfur* to the skin of head and neck AD patients can elicit an eczematous reaction with CD4+ T-cell and eosinophil infiltration (Tengvall Linder et al., 2000). Compared to healthy controls and SD patients the host's SIS response to *M. furfur* in AD exacerbates disease.

Investigators have tried to establish how *M. furfur* alters the immune system in AD, or more precisely how *M. furfur* further interacts with the altered abnormal immune response of the AD patient. As IgE accumulation is observed in biopsied AD affected skin, it comes as no surprise to detect elevated overall IgE antibodies in the serum of AD patients. High

levels of anti-*M. furfur*-specific IgE antibodies are detected almost exclusively in AD patients and it has been postulated that a T-cell dysregulation is responsible for this hyperstimulation of the IgE response. Although admittedly tenuous, T-cell depletion (CD8+ in AD patients) is a common theme in the ability of *M. furfur* to elicit disease (HIV+ve CD4 cell depletion). It is thought that CD8+ cell regulation of B-lymphocyte IgE production is depleted in AD resulting in the abnormal response of the AD skin to *M. furfur*. One study gives support to this theory of T-cell depletion, when peripheral blood mononuclear cells from AD patients showed increased production of IL-4 and IL-10 in response to *M. furfur* stimulation (Kroger et al., 1995). IL-4 is secreted by CD4+ Th2 cells and it stimulates B-cell secretion of IgE and eosinophil and mast cell proliferation. IL-10 is an 18kD protein produced by Th2 cells, CD8 T-cells and KCs. The most important activity of IL-10 is that it inhibits the production of cytokines by activated T-lymphocytes, inhibiting the Th1 cell response, tipping the immune balance in favour of the humoral immune response and hence stimulating IgE response. This may mean that in the normal host a T-cell response, specifically the Th1 response is integral in regulating the infectious capacity of *M. furfur*. The state of the *M. furfur* population, and the axis between *M. furfur* and *M. sympodialis* may be significant so perhaps *M. furfur* growth is encouraged by the lack of the Th2 response in AD. There is a significantly higher T-cell response of the Th2 variety in AD, (Tengvall Linder et al., 1996) and a distinct Th2 stimulatory cytokine profile is induced by the organism (Tengvall Linder et al., 1998). Hence the significance of the Th2 response appears to be relevant in the regulation of the immune privilege that exists for *M. furfur*. It is interesting to note that HIV infection, while directly affecting CD4 + T-cells also infects LCs; hence the ability of *M. furfur* to be presented as antigen to the efferent lymph nodes. If a dysregulation in the T-cell response results in infection, there should be a detectable difference in the humoral immune response of the normal infected population in AD and PV, which is not the case. Hence the abnormal AD patient may have a distinct novel immune response to *M. furfur*.

5.2.6 PSORIASIS

Psoriasis is an inflammatory scaly dermatosis which affects approximately 2% of the population. Abnormalities in several genes have been detected, many involving the HLA loci, indicating abnormalities in the host immune system for disease pathogenesis. The psoriatic patient can therefore be considered an immune abnormal host, hence the reaction

of the psoriatic skin to *M. furfur* is curious. Firstly the addition of heat-killed *M. furfur* to the psoriatic skin results in the initiation of a psoriatic-like lesion (Lober et al., 1982), indicating the ability of heat-killed organisms to exacerbate the abnormal skin. Some correlation between *M. furfur* and head and face psoriasis has been detected (Rosenberg et al., 1989) and while the hypothesis that microorganisms interact in psoriasis is not novel (Noah, 1990) the interaction of *M. furfur* is fairly new. A *M. furfur*-specific IgG response is detected in 73% of psoriatics (Squiquera et al., 1994) and this response is directed against the N-acetylglucosamine component of the fungal cell wall (Mathov et al., 1996). This agrees with the Malf 2 and Malf 3 proteins, which react with serum-specific IgE isolated from AD patients (Schmidt et al., 1997). These proteins have sequence homology with two *Candida biodini* membrane proteins, and while not cell wall-associated these proteins may come into contact with the host during replication. The Malf 4 protein however is known to be homologous to mitichondrial malate dehydrogenase, and the relevance of this homology is unknown.

While the humoral response is significant in the clearance of any infection, neutrophil migration and the T-cell response is perhaps more significant in psoriasis. The protein component of *M. furfur* significantly enhances polymorphonuclear neutrophil migration and this effect is specific for *M. furfur*, as opposed to other skin commensals like *S. epidermidis* (Bunse and Mahrle, 1996). T-cells from individuals with psoriasis of the head initiate a Th2 CD4 + T-cell response which is dependent on HLA-DR binding, with no difference between cytoplasmic or cell wall extracts of *M. furfur* in stimulating the immune response. All T-cells respond more significantly to the cytoplasmic extracts of *M. furfur;* however this response was not different to the normal scalp-derived T-cell lymphocytes and psoriatic scalp derived T-cells (Baker et al., 1997). Hence little evidence as to how *M. furfur* interacts with the psoriatic immune system exists. It does appear however that *M. furfur* possesses the ability to stimulate neutrophil migration and the KC studies indicate that KC may be the source of IL-8; however the actual interaction of *M. furfur* with the psoriatic inflammatory mechanism is unknown.

In summary, there is no conclusive evidence which pinpoints either an organism or host-derived cator which may predominate in *M. furfur* infection, either in normal infected individuals and psoriatics/ AD patients. One area that has received little experimental

investigation is host differences in the innate immune response of the skin to *M. furfur*. Due to the immense cytokine production profile of KCs, it is not unreasonable to assume that alteration of an individual's innate SIS homeostasis may affect the pathology of *M. furfur* infection in the 'normal' (non-AD/psoriatic) infected host.

5.3 INNATE IMMUNE RESPONSE

The capacity of *M. furfur* to interact with the innate component of the SIS has received little attention. However, it is reasonable to hypothesise that differences in host innate immunity to *M. furfur* may be responsible for disease pathology. It is possible that innate immune dysregulation could directly activate the pathology seen in *M. furfur* infections. The pathology associated with both PV and SD are perhaps the two most widely studied of the infections and despite their common origin, the pathology of the diseases is different. Enhanced pigmentation, increased sebum production, mild cellular infiltrate, and hyperproliferation are all symptoms of these infections and could be directly linked to an alteration of the skin's innate immune response. To consider the SIS as an axis, particularly in relation to IL-1α is relevant. The skin, in particular the epidermis, like any other organ maintains a homeostasis which is controlled by the differentiation of its cellular components and its interaction with the outside world. Differentiation of KCs is controlled by a myriad of factors including cytokines, with IL-1α in particular controlling terminal cornification of KCs. Disruption of the biological status of the skin results in disruption of the differentiation of the skin, and much of the pathology associated with *M. furfur* infections is associated with a disturbance of the homeostasis of the skin by the organism, or organism-derived components. Hyperproliferation, neutrophil migration and vasoconstriction are all associated with fungal infections; hence knowledge of the interaction of *M. furfur* with components of the innate SIS should clarify whether the organism can push the homeostatic balance of the skin from normal to diseased pathology.

5.3.1 COMPLEMENT FIXATION

M. furfur is able to fix complement via C3 and factor B, and as KCs produce both C3 and factor B *M. furfur* could theoretically stimulate the immune system via the alternative pathway of complement fixation. This could initiate the inflammatory response of the skin, and additional injury of the skin by complement activation could exacerbate diseases

already present, such as AD and psoriasis. This complement fixing activity is common to other yeast species, including *Candida,* and dermatophytes have also exhibited the ability to initiate the complement fixing immune response. While the efficacy of complement activation in clearing cutaneous fungal infections is questionable, the inflammation caused by lysis of yeast, albeit limited, may push the SIS axis over the homeostatic edge. Further studies into the potential of complement fixation to alter the skin homeostasis should be undertaken to elucidate the relevance of complement fixation in the innate immune response.

5.3.2 DEFENSIN

No previous study has investigated either the production of HBD-2 in a range of skin models or the production of HBD-2 in response to *M. furfur*. The work discussed here provides inconclusive evidence that fungal growth can stimulate HBD-2 expression on skin models. As measured by both the SEM/histological data and *M. furfur* growth curves, *M. furfur* growth on both LSE and BRT was slow but stable. Allthough it was not possible to test the antifungal ability of HBD-2 expression to suppress *M. furfur* growth, it is reasonable to assume that it has the same action on *M. furfur* as it does on *C. albicans* (Harder et al., 1997). While the results reported here were neither consistent or statistically significant it is tempting to speculate that when *M. furfur* is establishing gowth (in the first 24hr on the LSE and BRT) it does not stimulate HBD-2 production. This would suggest that different phases of *M. furfur* growth may result in different antigen expression. It is possible that if some populations of *M. furfur* remain very slow growing, then they will not stimulate HBD-2 expression and would possibly not stimulate cytokine production. As discussed these experiments did not provide consistent results hence perhaps the most important aspect of this work is the detection of a large range of HBD-2 expression between donors, indicating widespread differences in the magnitude of HBD-2 expression. Despite the unreliable results obtained from the experimental work performed it is tempting to speculate about the effect of the apparent suppression and stimulation of HBD-2 expression in the skin models. It is possible that the individual's different basal expression of HBD-2 could alter *M. furfur* colony growth, and consequently alter the metabolism of the organism on the skin. Thus, while HBD-2 expression alone cannot alter the pathology of the skin, it may exert some influence on the growth pattern of the organism on the skin. If the organism grows in a more antigen-producing population on the

skin, then HBD-2 expression could inadvertently contribute to the initiation of pathology in *M. furfur* disease. It would be interesting to investigate this tenuous link by firstly examining metabolite production by *M. furfur* both in culture and on the skin models, to correlate the production of antigen with *M. furfur* stimulation. If a connection were detected then it would be logical to examine the expression patterns of HBD-2 in *M. furfur*-infected individuals, to determine if there is any association between *in vitro* and *in vivo* patterns. It would be beneficial to the overall investigation of *M. furfur* biology and the SIS to identify if it is indeed a PPPR that transduces the signal for HBD-2 expression, or IL-1α expression, and the actual PPRR ligand component which originates from *M. furfur* and *C. albicans* which activates this receptor. It may be that *M. furfur* growth is selected over *M. sympodialis* in some individuals; perhaps if HBD-2 is more toxic/ antifungal against *M. furfur* then defensin production might alter growth of the commensal *Malassezia* population toward the pathogenic *M. furfur*. Only yeasts were tested in these models; hence, how the LSE, KC and BRT response to other commensals such as bacteria (*S. epidermidis*) may reveal a yeast-specific regulation of colonisation. Finally it should be remembered that these results were not standardly reproduced. Therefore it would be necessary to establish these trends in a larger population size, and examine their relative relevance in terms of how HBD-2 production affects *M. furfur* colonization before further examining the role of HBD-2 production in controlling *M. furfur* infection.

5.3.3 CYTOKINES

Some studies have investigated the cytokine response of various cells to *M. furfur*. Only one published study indicates that *M. furfur* can directly down-regulate the immune response by suppression of IL-1β production by peripheral blood mononuclear cells, while the IL1-α expression is unaltered (Walters et al., 1995). Other studies have shown how *M. furfur* induces granulocyte and macrophage production of IL-1α and IL-8 and opsonisation of *M. furfur* is shown to induce stronger expression of IL-8 mRNA (Suzuki et al., 2000b). No study apart from that carried out by Walters et al has investigated the innate cytokine profile of KCs. As discussed, the cytokine profile of the KC is immense and alteration, however small, in the homeostasis of the skin stimulates pathological changes in the skin. It is possible that host differences in cytokine reaction to *M. furfur* may push the skin homeostasis over the edge into disease pathology. Several studies could be considered to address these questions. Firstly, it would be interesting to examine IL1α production by infected donors both from involved and non-involved sites and compare this to controls. In

addition, further semi-quantitative RT-PCR on both LSE and BRT colonised with *M. furfur* could be carried out to compare the KC skin model cytokine mRNA production with both the BRT and LSE. It would also be interesting to test the reaction of the KCs to both cytoplasmic extract and viable *M. furfur* cells in an attempt to clarify the stimulatory element of the organism.

Cytokines, and in particular ET-1, may have a direct effect on melanin synthesis in the tissue and it would therefore be logical to assess the effect of anti-cytokine antibodies of melanin production. However as the IL-1α axis is the main homeostatic cytokine of the skin, any release of IL-1α, either via KC storage of the preformed cytokine or via targeted release, greatly alters the pathology of the skin. In addition to directly stimulating KC proliferation and MC melanin synthesis, IL-1α also amplifies other cytokine responses of the skin; thus it may amplify other reactions to the organism. One interesting point to note on IL-1 α production is how it affects sebum production. IL-1α can stimulate both KC proliferation, seen in many *M. furfur*-associated diseases, and increased sebum production. In this work IL-1α production by KCs was measured in response to *M. furfur* cell walls. The growth phase of *M. furfur* can have an effect on the immunostimulatory capacity of the organism. Here, freeze-dried cell wall isolated from organisms in the exponential phase of growth was used. This cell wall contains glucan, lipid and mannan which would of course be present when the organism is growing on skin. The cell wall was shown to significantly increase IL-1α production on KC monolayers. This result indicates the primary cytokine stimulation capacity of cellular components of *M. furfur*, which could account for the hyperproliferation seen in the SD epidermis. The cellular response in PV is of a mature cell type response with moderate cell infiltrates in both epidermis and dermis as compared to biopsies from normal-looking skin (Scheynius et al., 1984).

5.4 SUMMARY

By examination of the published data it is evident that little agreement on the pathogenic mechanisms of *M. furfur* exists. Past evidence indicates that differences in the host reaction to *M. furfur* could be responsible for some of the pathology associated with *M. furfur* disease. These studies demonstrate that host differences in the innate immune response of the skin to the organism exist. Differing levels of basal HBD-2 expression exists between donors and this could influence the growth of the *Malassezia* populations on the skin. In

addition *M. furfur* stimulates cytokine production by KCs indicating its ability to directly alter the proliferation of the skin (IL-1α), the *M. furfur* specific antibody production (IL-8) and the melanin synthesis of the skin (ET-1). *M. furfur* metabolism also directly stimulates melanin synthesis thus indicating a mechanism by which the organism could alter the pigmentation of the skin. In addition these studies demonstrate that host tissue, and putatively host lipid content, greatly affects the growth and morphology of *M. furfur* on skin, suggesting that the stimulation of certain growth patterns on skin may contribute to the pathogenicity of the organism.

In closing while no singularly definitive host-derived factor can be identified, these studies indicate that the range of host reactions to *M. furfur* may well contribute to the establishment and progression of *M. furfur*-associated infections.

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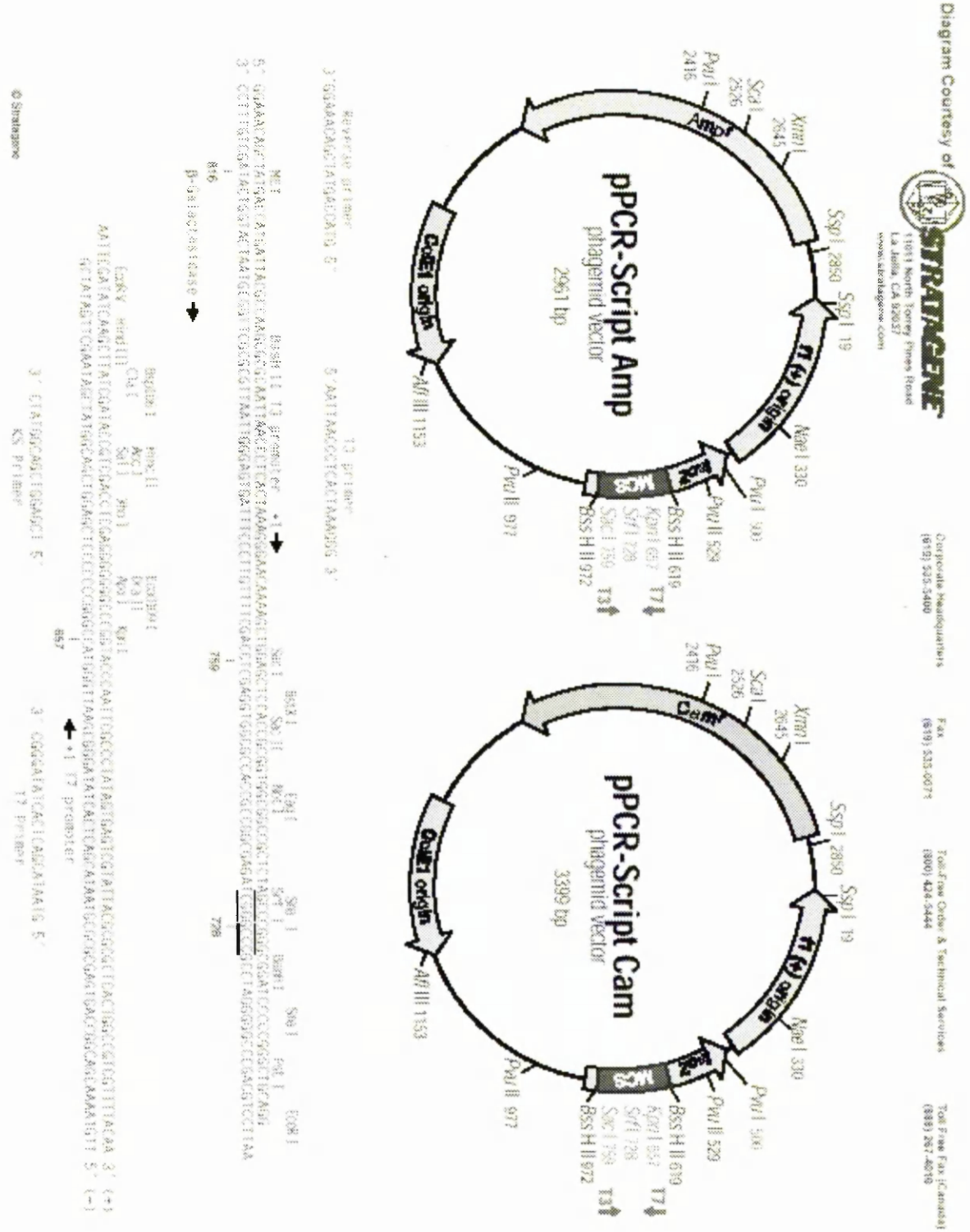
7 APPENDIX

7.1 APPENDIX FIGURE 1



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