School of Biomedical Sciences

CYTOKINE GENE EXPRESSION PATTERNS AND IMMUNE RESPONSES TO SYSTEMIC *CANDIDA ALBICANS* INFECTION IN INBRED MICE

ALEC JAMES REDWOOD B.App.Sci (Hon)
Curtin University of Technology

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DECLARATION

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a high degree to any other University or Institution.

Alec Redwood

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TERMINOLOGY

Colonisation

The yeast load of tissues, as determined by quantitative organ culture, is frequently referred to as colonisation in this Thesis. Colonisation or tissue colonisation was chosen to describe yeast load as it is a term widely used in the *Candida* literature. Colonisation is not used in this Thesis to imply *C. albicans* growth without concomitant tissue damage. Colonisation levels are expressed as $\log_{10}$ colony forming units/gram (cfu/g) of tissue. In some cases this has been abbreviated to logs.

Systemic candidiasis

The term systemic candidiasis is used in this thesis in preference to haematogenous or disseminated candidiasis and is used to indicate the systemic dissemination of *Candida* sp through the blood stream to single or multiple organs. The term candidiasis is used instead of candidosis.

Th1/Th2 cytokines

In 1986 Mosmann and colleagues described the presence of two subsets of Th cells from long term murine T-cell clones, which he termed Th1 and Th2 cells. Th1 cells secrete IL-2, IFNγ and TNFβ. Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 (Mosmann *et al.*, 1986; Howard *et al.*, 1993). It is becoming common in the literature to describe cytokines, such as IFNγ, as a Th1 cytokines and cytokines such as, IL-4 and IL-10, as Th2 cytokines. This is despite the fact that in some cases the origin of the cytokine is unknown or it has not been proven to be derived from a T-cell of that particular phenotype. IL-12, which stimulates the expression of a Th1 response, is also referred to as a Th1 cytokine even though it is not know to be produced by T-cells. Hence, it is common in the literature to call any cytokine that is known to stimulate the production of a Th1 response and/or inhibit the generation of a Th2 response, a Th1 cytokine regardless of the cell type producing it. The opposite can be said for Th2 cytokines. Whilst these terminologies may not be strictly correct, as the origin of the cytokine is unknown, they are used in this Thesis for the sake of simplicity and to indicate the type of immune response these cytokines may engender. Hence, in this Thesis, a "Th1 cytokine" is a cytokine that is known to inhibit a Th2 response and/or enhance a Th1 response. The term Th2 cytokine is used in a similar fashion.

When discussing overall responses, were the origin of the cytokine mRNA species is unknown, the terminology "type 1/2" or "Th1/2 like" response has been employed. These terminologies are equivalent to the Th1/Th2 paradigm, respectively, in terms of the broad immune responses. While it may be accurate to describe cytokines as "Th1/2" because of their effects on the generation of Th-cell phenotype it was not considered accurate to describe the overall immune response in these terms. Hence, It was felt that the rather loose description of "Th1/Th2" could not be applied to the general immune response.

ABBREVIATIONS

α	alpha
Ab	antibody
ADCC	antibody dependent cellular cytotoxicity
Ag	antigen
AGE	agarose gel electrophoresis
AIDS	acquired immune deficiency syndrome
APC	antigen presenting cell
β	beta
bp	base pair
C'	complement
C. albicans	*Candida albicans*
cAMP	cyclic adenosine monophosphate
CBA	CBA/CaH
cDNA	copy DNA
cfu/g	colony forming unit/gram
cm	centimetre (s)
CMC	chronic mucocutaneous candidiasis
CMI	cell mediated immunity
cpm	counts per minute
cRNA	copy RNA
CsCl	caesium chloride
CSF	colony stimulating factor
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytidine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxy guanosine triphosphate
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
DTH	delayed type hypersensitivity
DTT	dithiothreitol
dTTP	deoxy thymidine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylene diamine-tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EtBr	ethidium bromide
G-CSF	granulocyte-colony stimulating factor
γ/g	gamma/gram (s)

GM-CSF	granulocyte/macrophage-colony stimulating factor
H&E	haematoxylin and eosin
HCl	hydrochloric acid
HgO	mercuric chloride
HIV	human immunodeficiency virus
hpH_2O	Millipore high pure water
HSP	heat shock protein
Hx	haematoxylin
IFNγ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
iv	intravenous
L. major	*Leishmania major*
logs	log_{10} cfu/g
M, mM, μM, pM	molar, millimolar, micromolar, picomolar
M-CSF	macrophage-colony stimulating factor
mAb	monoclonal Ab
MAN	mannan
mg, μg, ng, pg, *fg*	milligram, microgram, nanogram, picogram, femtogram
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
ml, μl	millilitre, microlitre
MOPS	3-[N-morpholino]-propane sulphonic acid
mRNA	messenger ribonucleic acid
N_2	Nitrogen
NH&MRC	National Health and Medical Research Council
NK cell	natural killer cell
o/n	over night
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid shiffs
PBM	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
PMN	polymorphonuclear neutrophil
RE	restriction endonuclease
RNA	ribonucleic acid

RNase	ribonuclease
RT	reverse transcriptase/room temperature
SA-HRP	horse radish peroxidase conjugated streptavidin
SAB	sabourauds agar
SCID	severe combined immunodeficiency syndrome
SEM	standard error of the mean
SQ-RT-PCR	semi-quantitative reverse transcriptase PCR
ssDNA	single stranded DNA
TAE	tris acetate EDTA buffer
TE	tris-EDTA
TGFβ	transforming growth factor-beta
Th	T helper (cell)
tm	melting temperature
TNFα	tumor necrosis factor alpha
Tris	tris(hydroxymethyl)aminomethane
UV	ultra violet
V	volts
YEPD	yeast extract peptone dextrose (broth)
°C	degrees Celsius

CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

Fungi are eukaryotic organisms with approximately 300 000 different species. Of these, about 200 are potential parasites, with only a few of these affecting humans (Richardson, 1991). Fungal diseases, mycoses, range from the common mild deep cutaneous or subcutaneous skin infections, to the potentially lethal acute or chronic infections of deep tissues. These mycoses can be caused by pathogenic saprophytes from the environment or opportunistic pathogenic fungi which are part of the normal flora of the host organism.

Human opportunistic infections are mostly caused by yeasts of the genus *Candida*, by *Cryptococus neoformans*, five *Aspergillus* sp, and several of the zygomcetes (Muller, 1989). Of the *Candida* sp afflicting humans, *C. albicans* is by far the most common (Finegold and Baron, 1986). However, in recent years other *Candida* sp are being isolated as a cause of candidiasis with *C. tropicalis* being the next most common. Other less common species include *C. kruseii, C. parapsilosis, C. stellatoidea, C. guilliermondii, C. lusitaniae* and *C. pseudotropicalis.*

1.2 *CANDIDA ALBICANS*

C. albicans is a diploid, dimorphic fungi, existing in a yeast form or in a pseudohyphal form. It is an opportunistic fungal pathogen that is found as part of the normal flora in the gastrointestinal tract, upper respiratory tract and in the genital tract. Generally its growth is inhibited by other commensal organisms and the host immune system.

C. albicans is most commonly associated with the disease thrush. Thrush has been described as far back as the fourth century B.C. Hippocrates described two cases of oral aphathae, probably oral candidiasis, which were related to underlying disease. The causative agent of thrush was first described by Langenbeck in 1839 when he incorrectly assumed the fungus causing oral thrush in a typhus patient was the aetiological agent of typhus. Gruby in 1842 defined oral candidiasis and its causative agent. Wilkinson in 1849 was the first to describe vaginal thrush and in 1875 Haussman demonstrated that oral and vaginal thrush were caused by the same organism. Systemic candidiasis was first described in 1866 by Zenker in a debilitated patient with oral thrush who subsequently developed brain lesions.

1.3 *CANDIDA* PATHOGENESIS

The severity of infection with *C. albicans* is often a function of the host immune defect. However, there are differences in the pathogenicity of *C. albicans* strains which suggest that strain related virulence factors may play a role in disease severity. Numerous virulence factors have been attributed to the pathogenicity of *C. albicans*. These include adherence, dimorphism (pseudohyphae production), phenotypic switching, immune interference and protease production.

1.3.1 Adherence

Adherence of the yeast to mucosal epithelial cells is a necessary component of the infective process with *C. albicans*. Low adherent strains of *C. albicans* are less virulent in animal models (Calderone *et al.*, 1985; Ollert *et al.*, 1990). Invasive strains of *C. albicans* recovered from patients show greater adherence than strains recovered from carriers (McCourtie and Douglas, 1984).

Attachment is believed to be due to a specific interaction of *C. albicans* cell wall components and the target surfaces (McCourtie and Douglas, 1984). Cell surface mannoproteins have been linked to this attachment (Lee and King, 1983; Calderone *et al.*, 1988; Calderone and Wadsworth, 1988; Calderone and Braun, 1991) as have glycosides (Critchley and Douglas, 1987). Factors that enhance adherence include, fungal cell surface hydrophobicity, the phenotype of *C. albicans*, pH, temperature, pregnancy, diabetes, and oral contraceptive usage (Hazen, 1990; Vartivarian and Smith, 1993).

1.3.2 Dimorphism

Several studies suggest that the ability of *C. albicans* to switch between the yeast and mycelial forms is an important virulence factor. Increased adherence to oropharyngeal surfaces has been noted for the mycelial form (Kimura and Pearsall, 1978). Decreased adherence has been demonstrated by a non-germ tube producing variant in experimental vaginitis (Sobel *et al.*, 1984). The mycelial growth form has increased resistance to phagocytic killing by granulocytes and monocytes (Schuit, 1979; Cutler and Poor, 1981). The mycelial phase is also believed to be more invasive than the yeast phase (Evans, 1980). Antigenic differences exist between the two growth forms (Ashman *et al.*, 1990). These antigenic differences may influence the type immune response generated to *C. albicans* or may enhance immune evasion. Many of the putative virulence factors are expressed only on the mycelial growth form (Ashman *et al.*, 1990; Mayer *et al.*, 1990). Hence, the mycelial form is often considered to be the pathogenic form. However, mutant strains of *C. albicans* which are unable to switch into one form or the other, do remain pathogenic (Shephard, 1985). Both growth forms can be seen in infected tissue (Evans, 1980).

1.3.3 Phenotypic switching

C. albicans has the ability to undergo phenotypic switching both *in vitro* and *in vivo* (Slutsky *et al.*, 1985; Slutsky *et al.*, 1987; Soll *et al.*, 1987). These phenotypic differences are a product of differences in surface protein expression (Anderson *et al.*, 1989). The different surface protein expression results in differential adherence characteristics for the switch variants (Kennedy *et al.*, 1988) and differential sensitivities to neutrophil killing (Kolotila and Diamond, 1990). These data suggest, that differences in the phenotype may allow for increased resistance to immune attack or increased invasivness.

1.3.4 Molecular mimicry

The ability of *C. albicans* to mimic certain components of the immune system may enhance its virulence. A monoclonal antibody to a human monocyte antigen CD33, a differentiation/maturity marker, cross reacts with an antigen on the surface of *C. albicans* germ tubes (Mayer *et al.*, 1990). *C. albicans* has receptors for the activated components of complement C3, iC3b and C3d corresponding to the human complement (C') receptors CR3 and CR2 (Heidenreich and Dierich, 1985; Vartivarian and Smith, 1993). The human C' receptors CR2 and CR3 are members of the integrin family and are themselves adherence molecules. By binding iC3b, *C. albicans* may impair phagocytic uptake and killing. Additionally, the iC3b receptor increases the adherence of *C. albicans*. Antibodies to CR3, the human neutrophil receptor for iC3b, or to iC3b itself, inhibit adherence of *C. albicans* to human and animal endothelial cells (Frey *et al.*, 1990). In addition, an avirulent mutant strain of *C. albicans* lacked or had reduced levels of this iC3b receptor (Ollert *et al.*, 1990).

Activation of the alternative pathway of C' may enhance *C. albicans* growth by increasing the potential to obtain iron from host red blood cells. Complement

deposition on red blood cells allows the yeast to bind red blood cells via the C' receptors. This binding allows for the acquirement of iron which is necessary for *C. albicans* growth (Moors *et al.*, 1992). *C. albicans* can increase the production of C' factors C3 and B by human macrophages *in vitro* (Hogasen and Abrahamsen, 1993), possibly further enhancing immune evasion or iron uptake (Moors *et al.*, 1992).

A variety of protein binding sites have been identified on the surface of *C. albicans* germ tubes. These include sites for, albumin, fibronectin, lamin, transferin and fibrinogen (Hostetter, 1994). Steroidal binding sites have also been identified on the surface of *C. albicans* (Loose and Feldman, 1982; Powell and Drutz, 1983; Powell and Drutz, 1983; Das and Datta, 1985; Skowronski, 1989). The ability to bind human proteins and steroids may enable *C. albicans* to evade immune recognition. Binding these moieties may also serve as a means of acquiring growth factors from the host, such as iron (Moors *et al.*, 1992), or growth signals, such as oestrogen (Tanka *et al.*, 1989). Of importance is the fact that most, if not all, of these binding sites are found only on the surface of the germ tube/pseudohyphal stage of growth.

1.3.5 Extracellular virulence factors

C. albicans does not produce any known endo or exotoxin. However, *C. albicans* does produce a range of substances that may be important virulence factors. Protease production has been linked to increased virulence of *C. albicans* (MacDonald and Odds, 1980; Kwon-Chung *et al.*, 1985). The increased virulence of protease producing strains may be due to an enhanced ability to invade tissues (MacDonald and Odds, 1983; De Bernardis *et al.*, 1990), or to other factors, such as, increased adherence (Borg and Rüchel, 1988; Edison and Manning-Zweerink, 1988) or resistance to phagocytosis (Kaminishi *et al.*, 1995).

Other factors produced by *C. albicans* may inhibit the host immune response. Diamond and colleagues (Diamond *et al.*, 1980b; Smail *et al.*, 1992) identified several substances produced by *C. albicans* that prevented contact between the fungi and human neutrophils and inhibited neutrophil function. Further, *C. albicans* produces a factor that is able to inhibit the release of azurophil and specific granules by human neutrophils (Smail *et al.*, 1989) and inhibit the respiratory burst of human neutrophils (Smail *et al.*, 1988). Finally, considerable evidence exists for the immunosuppressive effects of *C. albicans* components such as mannan (Garner *et al.*, 1990).

1.4 CANDIDIASIS

C. albicans produces a range of infections in the normal and immunocompromised or immunosuppressed patient. These can be defined in two broad categories, superficial mucocutaneous and systemic invasive. Superficial infections affect the various mucous membrane surfaces of the body such as in oral and vaginal thrush. Superficial infections generally are benign or at least not life threatening. In contrast, systemic infections, involving the spread of *C. albicans* to the blood (candidemia) and or the major organs, are frequently fatal. Systemic infections can manifest as single organ involvement, or as disseminated candidiasis which encompasses a range of organs.

1.4.1 Superficial candidiasis

1.4.1.1 Thrush

The incidence of vulvo vaginal candidiasis (thrush) has increased approximately 2 fold in the U.S.A in the last decade (Kent, 1991). Approximately 75% of women will experience at least one case of a clinically significant episode of vaginal thrush during their reproductive period (Hurley and De Louvois, 1979; Hurley, 1981). A significant percentage of these women, 40-50%, will experience a second attack (Hurley, 1981)

and approximately 5% will experience recurrent intractable episodes of vaginal thrush (Sobel, 1993).

Vaginal thrush is a relatively benign condition which responds well to anti-fungal treatment. It is believed the infection is due to minor changes in epithelial conditions, such as, pH, altered glucose/glycogen concentration, or changes to the epithelial integrity (Khardori, 1989). During pregnancy the risk of vaginal thrush increases, possibly due to changes in hormone production, leading to increased glycogen content in the vagina (Sobel, 1993).

Oestrogen is believed to play a role in susceptibility to vaginal thrush, both by increasing adherence (Sobel, 1993), as well as by inducing mycelial formation (Sobel, 1993). Oestrogen may induce mycelial transition by increasing cyclic AMP (cAMP) concentrations within the yeast (Tanka *et al.*, 1989). The oral contraceptive pill, which increases oestrogen levels, is a predisposing factor to vaginal candidiasis (Walsh *et al.*, 1968).

The pathogenesis of recurrent vaginal thrush may involve a defect in the local immunity to candidiasis possibly through inappropriate prostaglandin E_2 (PGE_2) production (Witkin *et al.*, 1986; Witkin *et al.*, 1988; Kalo and Witkin, 1990). Recurrent vaginal candidiasis is also common in female patients with acquired immune deficiency syndrome (AIDS), suggesting a role for depressed cell-mediated immunity in recurrent vaginal thrush (Sobel, 1993). Decreased levels of interferon-gamma (IFNγ) may have a role in increased susceptibility, due, both to the action of IFNγ in a functional immune response and to IFNγ mediated inhibition of germ tube formation (Kalo and Witkin, 1990).

Oral thrush and other superficial *C. albicans* infections are seen in a significant proportion of newborns, especially in infants requiring neonatal intensive care. Oral

thrush is seen in the majority of patients with AIDS, with a high proportion developing oesophageal candidiasis. The linkage of AIDS with oral thrush supports the hypothesis that T cells are critical components of the host defence mechanism to superficial infections. Oral thrush is common in patients with leukaemia, transplant recipients and patients receiving cancer chemotherapy (Roseff and Sugar, 1993).

1.4.1.2 Chronic Mucocutaneous Candidiasis

Chronic mucocutaneous candidiasis (CMC) is a more severe form of superficial candidiasis which can manifest as a variety of syndromes. It can manifest as a chronic infection of the skin, nails and/or mucous membranes. Patients with CMC have no predisposition towards systemic candidiasis. Syndromes of CMC include, chronic oral candidiasis, Chronic localised mucocutaneous candidiasis, CMC with thymoma, CMC with interstitial keratitis and CMC with polyendocrinopathy (Kirkpatrick, 1993).

The onset of CMC is usually associated with a defect in the cell mediated immune system and often presents with a variety of other infectious diseases (Herrod, 1990). Diseases that predispose to chronic mucocutaneous candidiasis include the endocrinopathies, such as juvenile hypoparathyroidism, hypoadrenalcortism and thymomas. Other diseases include Swiss type agammaglobulinemia and AIDS (Khardori, 1989).

Some patients develop chronic mucocutaneous candidiasis without apparent underlying disease. However, it is generally found that these patients do have some degree of T cell dysfunction (Kirkpatrick, 1993). In general, prolonged treatment with anti-fungals will clear CMC, however the disease will re-occur without correction of the underlying immune defect (Kirkpatrick and Smith, 1974).

1.4.2 Systemic Candidiasis

Systemic candidiasis is the dissemination of *C. albicans* through the bloodstream to the major organs. It can manifest as candidemia, single, or multiple organ involvement. Bodey *et al* (1993) defines haematogenous candidiasis as infections involving the bloodstream, which includes candidemia, disseminated candidiasis, or both. The term systemic candidiasis is used throughout this Thesis to describe haematogenous, and in particular, disseminated candidiasis.

Systemic candidiasis is largely a disease of iatrogenic cause, as the increased incidence of systemic candidiasis has been linked to advances in other areas of medical science. Before 1960 most medical literature focused on superficial candidiasis (Bodey, 1993). In 1959, Braude and Rock were able to find only four reported cases of systemic candidiasis. The advent of aggressive medical treatments such as chemotherapy and immunosuppression, has lead to a rise in systemic candidiasis. The widespread use of antibiotics has also increased the incidence of systemic candidiasis. In the 1950s 10% of leukaemia patients died from fungal infections (Bodey, 1966), this figure had risen to over 40% by the 1970s (Pizzo *et al.*, 1982). In each study *Candida* accounted for two thirds of the infections. Systemic candidiasis has been reported to be present in up to 25% of leukaemia patients (Bodey, 1993) and in 10-22% of bone marrow transplants recipients (Cliff, 1984).

Systemic candidiasis can involve any organ of the body, usually the kidneys, heart, gastrointestinal track and lung. Other organs affected can include the liver, spleen, central nervous system, lymph nodes and larynx. Systemic candidiasis most commonly affects at least three to four organs simultaneously (Bodey, 1993).

The pattern of organ involvement can indicate route of infection. Leukaemia patients frequently have gastrointestinal lesions from their treatment regimes. These patients

frequently have gastrointestinal, liver, spleen and lung involvement. This pattern of infection suggests spread of *C. albicans* from the GIT to the major organs via the portal circulation. Conversely other patients, such as surgical patients, have a much higher incidence of kidney and heart involvement, suggesting direct entry to the blood, probably via an intravenous catheter (Bodey and Anaissie, 1989; Myerowitz *et al.*, 1977)

1.5 PREDISPOSING FACTORS TO SYSTEMIC CANDIDIASIS

C. albicans is an opportunistic pathogen and as such produces disease in compromised individuals. Factors that predispose to systemic candidiasis include, disease related or induced immunosuppression, cancer, post-operative states especially complicated abdominal surgery, broad spectrum antibiotic therapy, low birth weight in neonates, burns, parental drug use, gastrointestinal pathology and hyperglycaemia.

Congenital diseases that predispose to systemic candidiasis are generally due to hereditary defects in neutrophil function such as, myeloperoxidase deficiency and chronic granulomatous disease (CGD).

Infection with the human immunodeficiency virus (HIV) produces a well characterised reversal of the CD4/CD8 ratio of T cells, due to a reduction in the number of $CD4^+$ cells. The immune defect produced by HIV predisposes to oral and oesophageal candidiasis in AIDS patients. However, these patients rarely develop systemic candidiasis, suggesting that T-cells are not involved in resistance to systemic candidiasis. Conversely, patients with leukemia, transplant recipients, and patients receiving cancer chemotherapy develop oral and systemic candidiasis.

Neutropenia, which is common in leukemia patients, is possibly the greatest predisposing factor to systemic candidiasis. The severity and duration of neutropenia

is closely related to the incidence of systemic candidiasis in patients with haematalogic malignancies and solid tumours (Maksymiuk *et al.*, 1984). The risk to bone marrow transplant patients is particularly high due to the severe neutropenia induced in these patients. The frequency of autopsy-proven fungal infections correlates well with the length of the neutropenia. Fungal infection was found in 21% of patients with neutropenia which had lasted 1-20 days, in 41% of those in whom it had lasted 21-40 days and 57% of those lasting more than 40 days (Bodey, 1966; Myers and Atkinson, 1983). The prognosis is also related to neutrophil count. Patients treated with amphotericin B had a 38% recovery rate if their neutrophil count was increased to normal during the therapy. However, those patients with persistent neutropenia ($<1.0x10^9/L$) had 0% recovery rate (Maksymiuk *et al.*, 1984).

Factors other than neutropenia, in transplant and other patients, predispose to systemic candidiasis. Transplant patients are usually treated with long term immunosuppressive therapy which depresses the resistance to systemic candidiasis. Prolonged use of moderate to high-dose renal corticosteroids is a common feature in patients with systemic candidiasis. Steroids decrease resistance to disease by impairing phagocyte function, suppressing acute and chronic inflammatory functions, retarding wound healing (barrier removal) and by suppression of cell-mediated and humoral immunity. Broad spectrum antibiotic therapy also predisposes to systemic candidiasis by depressing resident flora, which allows for the overgrowth of *Candida.* Additionally, some antibiotics, notably tetracycline, are capable of impeding neutrophil migration, phagocytosis and oxidative metabolism (Yourtee and Root, 1982) Antibiotic use is often superimposed onto underlying conditions of neoplasia and post-operative states. Typically a patient with systemic candidiasis has a chronic disease or has been subject to surgery or major trauma. Many have had severe and prolonged myelosuppression with neutropenia (lasting greater than 7 days). Most have been treated with broad spectrum antibiotics and immunosuppressives, in particular corticosterone. They have

one or more indwelling intravenous catheters and may be hyperglycemic due to diabetes or hyperalimentation.

1.6 HOST DEFENCE MECHANISMS TO SYSTEMIC CANDIDIASIS

1.6.1 Skin and Mucosal Barriers

The stratified squamous epithelium of the skin is a very effective barrier to invasion by *C. albicans*. Continuous desquamation ensures that *C. albicans* numbers are kept low and the presence of skin lipids inhibit growth of the yeast (Aly and Maibach, 1975). Mechanical breakdown of the skin is the most important predisposing factor for *Candida* skin infections (Vartivarian and Smith, 1993). This is seen in burns patients or when the skin is subject to increased moisture and maceration (Odds, 1988).

In contrast to the skin, mucosal surfaces are more readily colonised with *Candida* sp. Mucosal surfaces of the mouth, the gut and the vagina may be colonised by *C. albicans* in up to 80% of individuals (Gorbach, 1967; Odds, 1988). Factors that increase the numbers of *C. albicans* on mucosal surfaces, such as hospitalisation, diabetes and broad spectrum antibiotic therapy, may enable the yeast to switch from a commensal to a pathogen (Vartivarian and Smith, 1993).

1.6.2 Humoral immunity

The role of antibody (Ab) in resistance to systemic candidiasis remains contradictory. Anti-*Candida* IgA Ab in saliva has been shown to inhibit binding of *Candida* sp to the oral mucosa. However, as patients with candidiasis have higher secretory anti-*Candida* IgA than non infected controls, the role played by these Ab may be minimal (Epstein *et al.*, 1982). Diamond *et al* (1978) reported that small quantities of human

anti-*Candida* IgG Ab could enhance human neutrophil phagocytosis of *Candida* pseudohyphae.

The fact that dysfunctions of immunoglobulin production are not a predisposing factor to the acquirement of systemic candidiasis, coupled with the equivocal results obtained for specific Ab functional studies (Kagaya and Fukazawa, 1981; Kagaya *et al.*, 1981) suggests that Ab plays little role in the hosts defence against systemic candidiasis. Animal studies suggest that high Ab production may be linked to a poor immune response to systemic candidiasis (Romani *et al.*, 1991b), demonstrating a switch away from protective cell mediated responses.

1.6.3 Phagocytosis

Non-specifically activated polymorphonuclear neutrophils (PMN) are the primary phagocytic cells responsible for the clearance of *C. albicans* from infected tissues (Lehrer and Cline, 1971). Consequently neutrophilia is the most potent predisposing factor for the acquirement of systemic candidiasis (Bodey, 1966; Maksymiuk *et al.*, 1984). Human PMNs have been shown to phagocytose and kill *C. albicans* yeast cells and pseudohyphal forms (Verhaegen *et al.*, 1976; Diamond *et al.*, 1980a; Djeu *et al.*, 1986; Thompson and Wilton, 1992; Yee *et al.*, 1994). Human macrophages also have the ability to kill both yeast and pseudohyphal forms of *C. albicans* (Diamond and Haudenschild, 1981; Thompson and Wilton, 1992).

1.6.4 Cell Mediated Immunity

The aspect of the phagocytic system which functions as part of the innate immune system is hard to separate from that which is enhanced by cell mediated immune functions, such as, antibody dependent cellular cytotoxicity (ADCC) and cytokine promoted activation. Cell-mediated immunity can function in a variety of fashions.

Cytotoxic $CD8^+$ cells can mediate direct cytotoxicity to *C. albicans* (Beno *et al.*, 1995; Levitz *et al.*, 1995). Additionally, $CD4^+$ T-helper cells co-ordinate an array of functions including Ab production, $CD8^+$ help and perhaps direct $CD4^+$ mediated killing.

Whilst neutrophils are the most important effector cell in the defence from systemic candidiasis, the candidacidal activity of macrophages and neutrophils can be enhanced by various cytokines (Djeu *et al.*, 1986; Vecchiarelli *et al.*, 1989; Wang *et al.*, 1989b; Djeu *et al.*, 1990; Smith *et al.*, 1990; Marodi and Johnston, 1993; Stevenhagen and van Furth, 1993). An example of this may be occurring in AIDS patients. Systemic candidiasis occurs in approximately 1% of AIDS patients (Whimbey *et al.*, 1986), in most cases this appears to be due to the presence of other risk factors (section 1.5). However, systemic candidiasis has been reported in AIDS patients that have no known risk factors (Whitbey *et al.*, 1996). Impaired neutrophil function has been demonstrated in AIDS patients (Tachavanich *et al.*, 1996: Vecchiarelli *et al.*, 1995: Ellis *et al.*, 1988). It is not unreasonable to speculate that impaired neutrophil function is a result of a reduction in CD4 cells and hence T-cell derived cytokines. In which case, systemic candidiasis in the occasional AIDS patient may be indirectly related to decreased cell mediated immunity. Hence, T-cells may have direct and indirect effects on the growth of *C. albicans* and may contribute to innate mechanisms in the resistance to systemic candidiasis.

1.7 DIAGNOSIS AND TREATMENT OF SYSTEMIC CANDIDIASIS

There is no characteristic set of clinical signs and symptoms for the diagnosis of systemic candidiasis. The most common presentation is persistent fever that is unresponsive to broad spectrum antibiotics. Mental obtundation is present in 24% of patients at the initiation of disease (Bodey, 1993). Characteristic skin lesions are present in 10% of patients (Balandran *et al.*, 1973; Bodey and M., 1974). Some

patients present with fever and hypotension suggestive of endotoxic shock (Bodey, 1993). A significant correlation exists between multiple organ involvement and eye lesions. Endophthalamitis presents with a white discharge composed predominantly of PMN cells. Neutropenic patients, those at most risk of systemic candidiasis, do not produce this discharge (Bodey, 1993).

The lack of definitive signs or symptoms makes the diagnosis of systemic candidiasis difficult. Blood culture is often negative and the commensal nature of *C. albicans* makes false positives common. Due to the severity of side effects from anti-fungals, treatment is often delayed or not instigated due to lack of positive identification. The inability to diagnose systemic candidiasis pre-mortem, results in lack of anti-fungal therapy in many patients (Maksymiuk *et al.*, 1984).

Patient mortality can be as high as 80% among leukemia patients, 52% amongst burns patients and 63% among surgery patients (Roberts *et al.*, 1991). Although, the contribution of *C. albicans* to mortality is hard to assess as many patients are considerably compromised by underlying disease.

Conventional treatment for systemic candidiasis is the use of antifungals, in particular amphotericin B. However, many studies have failed to demonstrate an appreciable increase in survival rate in patients treated with antifungals (Horn *et al.*, 1985; Komshian *et al.*, 1988). This may be because the response to treatment is a function of the severity of the underlying disease (Komshian *et al.*, 1988). Mortality rates of 100% have been documented in patients treated with amphotericin B who have severe neutropenia, however when the neutrophil count returns to normal the mortality rate is reduced to 62% (Maksymiuk *et al.*, 1984).

1.8 MURINE MODELS OF SYSTEMIC CANDIDIASIS

The genetic and environmental diversity, and the difficulty in undertaking experimentation on humans, has led to the development of animal models of systemic candidiasis. Mouse models of systemic candidiasis demonstrate similar patterns of tissue invasion to those of humans (Anaissie *et al.*, 1993).

Genetically inbred strains of mice demonstrate different rates of resolution and sensitivity to systemic candidiasis (Hector *et al.*, 1982). Experimental models have exploited both naive and immune mice in an attempt to understand the immune effector mechanisms responsible for resolution of both primary and secondary systemic candidiasis (Hector *et al.*, 1982; Ashman and Papadimitriou, 1987; Ashman and Papadimitriou, 1988). Models of infection have also focused on the different growth forms of *C. albicans* (Bibas Bonet de Jorrat *et al.*, 1989). In general, studies of the immune responses in murine models of systemic and superficial candidiasis correlate well with clinical observations documented for human infections.

Hector *et al* (1982) described the sensitivity of six inbred mouse strains to systemic candidiasis; BALB/c, C57Bl/6J, DBA/2J, CBA, C3H and A/J by determining *C. albicans* numbers in infected tissue. Challenge of naive mice evidenced three patterns of resistance, BALB/c and C57Bl/6J mice were the most resistant. The CBA, C3H, and A/J mice demonstrated moderate resistance. DBA mice were the most sensitive to *iv* challenge. Ashman and Papadimitriou (1987) described the resistance patterns of three mice strains based on histology and tissue damage; BALB/c and C57Bl/6J mice were the most resistant, CBA/H mice showed the greatest tissue damage.

1.8.1 Murine Defence Mechanisms

1.8.1.1 Phagocytosis

The phagocytic system has long been considered the major host defence mechanism to systemic candidiasis. The PMN can ingest and kill *Candida* sp (Davies and Denning, 1972; Belcher *et al.*, 1973; Diamond *et al.*, 1978; Kagaya and Fukazawa, 1981; Levitz, 1992; Anaissie *et al.*, 1993). Killing is by both oxidative and non-oxidative mechanisms. In tissues, such as the kidney, the predominant inflammatory response to *C. albicans* is by PMN cells. Animal models have demonstrated a correlation between PMN infiltration with stabilisation of tissue numbers and resolution of the disease (Anaissie *et al.*, 1993).

Macrophages, like PMN, appear to be important in the host defence against *C. albicans* and can ingest and kill *Candida* (Lehrer, 1975; Leijh *et al.*, 1977; Evron, 1980; Diamond and Haudenschild, 1981; Kagaya and Fukazawa, 1981). Mice that have been depleted of splenic macrophages are more susceptible to systemic candidiasis (Qian *et al.*, 1994). Others have postulated that poor intracellular killing of *Candida* by macrophages may allow dissemination of the yeast through the tissues, with the macrophage serving as a vector (Arai *et al.*, 1977; Sugar *et al.*, 1983; Domer and Carrow, 1989). However, similar results have been obtained with polymorphonuclear leukocytes (Louria and Brayton, 1964; Belcher *et al.*, 1973). Hence it appears likely that both PMN and macrophages contribute to the non-specific immunity to systemic candidiasis. However, macrophages may take longer to become highly candidacidal than PMN (Calderone and Sturtevant, 1994).

1.8.1.2 Humoral Immunity

The role of humoral immunity in animal models of systemic candidiasis has yet to be fully defined. Passive transfer of a monoclonal Ab (mAb) to a major reactive epitope of *Candida* heat shock protein (HSP) 90 protects against systemic challenge with *C. albicans* (Matthews *et al.*, 1991). *C. albicans* specific Ab can enhance neutrophil and macrophage killing of *C. albicans* (Khardori, 1989). However, the transfer of immunoglobulin from rabbit anti-*C. albicans* serum enhanced resistance to *C. albicans* only in delayed type hypersensitivity (DTH) positive mice (Kagaya *et al.*, 1981). Therefore, the role of humoral immunity in animal models of systemic candidiasis remains inconclusive. The reciprocal regulation of Ab and DTH responses, discussed below, may indicate that Ab production is at best a moderating influence and at worst a contributor to disease susceptibility (Parish, 1972; Mosmann *et al.*, 1986; Finkelman *et al,* 1990; Romani *et al.*, 1991b; Romani *et al.*, 1993).

1.8.1.3 Cell Mediated Immunity (CMI)

There is considerable evidence to suggest that T-cells play little or no role in systemic candidiasis. Neither nude mice, nor adult-thymectomised, irradiated, bone-marrow reconstituted mice show an increase in susceptibility to candidiasis (Rogers *et al.*, 1976; Giger *et al.*, 1978; Tabeta *et al.*, 1984). Further, nude mice have been shown to be more resistant to systemic candidiasis than normal mice (Cutler, 1976; Rogers *et al.*, 1976), presumably due to an enhanced innate immunity (Cheers and Waller, 1975; Zinkernagel and Bladden, 1975). Finally, severe combined immunodeficiency syndrome (SCID) mice are not more sensitive to systemic candidiasis than normal mice (Mahanty *et al.*, 1988).

In contrast New Zealand black mice, which have a relative deficiency in CMI, are highly susceptible to systemic candidiasis (Corbel and Eades, 1976). The absence or

presence of DTH correlates well with mortality in immunised mice rechallenged with systemic candidiasis (Kagaya *et al.*, 1981). Additionally, resistant strains of mice have a greater DTH reaction to *C. albicans* and increased *Candida* specific lymphocyte proliferation (Ashman, 1990). T cell depleted mice are more susceptible to secondary candidiasis (Giger *et al.*, 1978). Depletion of $CD4^+$ cells prevents the development of increased resistance to secondary systemic candidiasis (Romani *et al.*, 1992b). In addition, nude mice have increased sensitivity to systemic candidiasis when the disease is allowed to run its full course, indicating the need for T-cell mediated immunity in the latter stages of the disease (Miyake *et al.*, 1978; Sinha *et al.*, 1987). Additionally, murine $CD8^+$ cells may be directly cytotoxic to *C. albicans* pseudohyphae (Beno *et al.*, 1995).

T cells specific for *C. albicans* have been used to protect sublethally irradiated mice from primary systemic candidiasis (Sieck *et al.*, 1993). Protection of mice from secondary infection with *C. albicans* is mediated by $CD4^+$ T helper (Th) cells (Cenci *et al.*, 1989). Hence, while some reports suggest that cell mediated immunity plays little role in the defence to systemic candidiasis, a large body of evidence indicates that this is not the case in secondary infection and later in primary infection.

Cytokines produced by T-cells and other cell types are important in the host immune response to systemic candidiasis. Phagocytosis and killing of *C. albicans* can be increased by a range of T-cell and non T-cell derived cytokines in both human and murine systems (Djeu *et al.*, 1986; Wang *et al.*, 1989b; Djeu *et al.*, 1990; Smith *et al.*, 1990; Roilides *et al.*, 1992; Stevenhagen and van Furth, 1993). Additionally, cytokines can mediate protection from systemic candidiasis *in vivo* (Pecyk *et al.*, 1989; Allendoerfer *et al.*, 1993; Kullberg *et al.*, 1993). Consequently, it is likely that the major role of T-cells in resistance to systemic candidiasis is in the production of a range of cytokine species that increase the candidacidal activity of neutrophils and macrophages.

In 1986 Mosmann and colleagues described the presence of two subsets of Th cells from long term murine T-cell clones. These clones differed in the patterns of cytokines they secreted. Mosmann and colleges described these cells as Th1 cells on the basis of IL(interleukin)-2, IFNγ and tumour necrosis factor-beta (TNFβ) secretion. Th2 cells were categorised on the basis of IL-4 and IL-5 secretion. Both cell types secreted IL-3, tumour necrosis factor-alpha (TNFα) and granulocyte/macrophage-colony stimulating factor (GM-CSF). The cytokine patterns produced correlate to a cell mediated DTH type of response for Th1 and humoral and allergic responses for Th2.

Since the initial classification by Mosmann and colleagues (1986) the pattern of cytokines secreted by Th2 cells has increased to include IL-6, IL-10 and IL-13 (Howard *et al.*, 1993). Other Th cell types, Thp and Th0 cells, have also been added to the list. Thp cells are putative precursor cells which progresses to the pre-committed Th0 cell. Th0 cells are able to produce a range of cytokines including simultaneous Th1 and Th2 patterns. It is not yet clear whether the Th0 cell is able to produce all the T cell cytokines or if different Th0 cells produce a different range of cytokines. In fact, it is not yet apparent that Thp and Th0 cells exist as discrete sets or subsets of cells, or that it is necessary for Th1 and Th2 cell to go through either of these intermediate cell types. However, it seems likely that T cells progress through some intermediate phase (such as Th0) that secrete a less restricted subset of cytokines, including IL-4, and then into a defined range of cytokine profiles, such as, Th1 and Th2 (Kamogawa *et al.*, 1993).

Cytokines produced by one subset of Th cells have the capacity to affect the generation and activation of Th cells from the opposing subset. While IL-4 does not inhibit the proliferation of Th1 clones, it does inhibit the generation of Th1 clones from naive cells as well as suppresses IFNγ production and DTH responses (Swain *et al.*, 1990; O'Garra and Murphy, 1994). Additionally, IL-10 can inhibit Ag specific T-cell

proliferation as well as inhibit cytokine production from Th1 clones (Howard and O'Garra, 1992). IL-10 also inhibits the production of IL-12 and down regulates B7 expression (O'Garra *et al.*, 1994). IL-12 is a macrophage derived cytokine that contributes to the production of a Th1 phenotype by its action of increasing IFNγ release form T-cells and NK cells (Seder *et al.*, 1993; O'Garra and Murphy, 1994). In the converse the "Th1" cytokine, IFNγ, can inhibit the proliferative responses of Th2 clones (Gajewski and Fitch, 1988). However, it appears that the Th2 response is dominant with an optimal Th1 response only being generated in the absence of IL-4.

The Th1 subset has been extensively linked to disease resistance in animal models, with leishmaniasis being the archetypal disease model. Mice resistant to cutaneous leishmaniasis, caused by *Leishmania major*, produce a protective Th1 response. Sensitive mice produce a non-protective Th2 response (Heinzel *et al.*, 1989; Scott *et al.*, 1989). This pattern of sensitivity and resistance has been demonstrated in other diseases such as leprosy (Salgame *et al.*, 1991; Yamamura *et al.*, 1991), schistosomiasis (Scott *et al.*, 1989) and AIDS (Clerici and Shearer, 1993). In general, Th1 responses are protective in models of infectious disease. However, in models of autoimmunity Th1 responses may be immunopathogenic (Scott *et al.*, 1994). The Th2 response is generally a non-protective response except in certain parasitic infections (Lange *et al.*, 1994).

Hector *et al* (1982) detailed different susceptibilities to *C. albicans* in six mouse strains and noted that the two most resistant strains, BALB/cyJ and C57Bl/6J, produced little Ab during infection. In contrast, the sensitive strains produced greater levels of Ab which had little protective effect in primary infection. Other data show that resistant mice have a greater DTH response than sensitive mice (Ashman, 1990). Therefore, these data suggest a Th1 response in mice resistant to systemic candidiasis and a Th2 response in mice sensitive to disease. The Th1 subset has been more conclusively linked to resistance to secondary systemic candidiasis by the work of

Romani *et al* (Romani *et al.*, 1991b; Romani *et al.*, 1992a), as has resistance to primary candidiasis (Romani *et al.*, 1993). Sensitivity was consistent with preferential activation or induction of Th2 cells in secondary (Romani *et al.*, 1991b) and primary systemic candidiasis (Romani *et al.*, 1992c; Romani *et al.*, 1993). The pattern of resistance and sensitivity described in systemic candidiasis fits the general description of protective Th1 and non-protective Th2. In this, IFNγ (Romani *et al.*, 1992c; Kullberg *et al.*, 1993) and IL-12 (Romani *et al.*, 1994a) have protective and opposing roles to IL-4 (Romani *et al.*, 1992c; Puccetti *et al.*, 1994) and IL-10 (Romani *et al.*, 1994b).

The mechanism for the switch to one or other Th type remains to be determined, although numerous hypotheses have been advanced; such as the antigen presenting cell (APC) type (Gajewski *et al.*, 1991; Pfeiffer *et al.*, 1991; De Becker *et al.*, 1994; Macatonia *et al.*, 1995; Secrist *et al.*, 1995), presence of competing cytokines (Swain *et al.*, 1990; Macatonia *et al.*, 1995; Mocci and Coffman, 1995), antigen (Ag) load (Parish, 1972; Bretscher *et al.*, 1992; Secrist *et al.*, 1995), Ag type (Pfeiffer *et al.*, 1991) presence of co-stimulatory molecules (Kuchroo *et al.*, 1995) and route of administration (Parish, 1972). It is likely that many of these factors operate in concert with each other (Macatonia *et al.*, 1995; Secrist *et al.*, 1995).

1.9 IMMUNOSUPPRESSION

Development of systemic or superficial candidiasis is generally due to a host immune defect or iatrogenic effects. The host immune defects have been well categorised and can be broadly defined as those predisposing to infection, or those that occur after infection. The occurrence of immune defects after infection suggests that *C. albicans* is capable of inducing some form of immunosuppression.

Recurrent vaginal candidiasis has been linked to a defect in T cell function (Witkin *et al.*, 1986). These defects in T-cell function may be a result of the infection, rather than a predisposing factor in the development of infection (Marmor and Barnett, 1968; Fidel *et al.*, 1993). Serum from patients with systemic candidiasis or CMC can inhibit T cell function (Canales *et al.*, 1969; Laforce *et al.*, 1975). The inhibitor of lymphocyte proliferation can be removed by treatment with amphotericin or plasmapheresis. Removal of the inhibitor correlates with recovery from disease and return to normal cell mediated immunity. In contrast, return of the inhibitor correlates with recurrence of the disease (Paterson *et al.*, 1971; Twomey, 1975).

A candidate for the suppressive factor produced by *C. albicans* is mannan (MAN) a component of the *Candida* cell wall (Fisher *et al.*, 1978). The mechanism of MAN induced suppression has been attributed to the production of PGE_2 by activated macrophages. (Witkin *et al.*, 1986; Nelson *et al.*, 1991). PGE_2 is a powerful immunomodulator that is produced by a variety of cells such as macrophages, dendritic cells and fibroblasts.

$CD8^+$ suppressor cells have been induced in CBA/J mice following injection with *Candida* MAN (Garner *et al.*, 1990). These cells when transferred to syngenic mice are able to mediate suppression of DTH. PGE_2 is able to stimulate the production of suppressor T cells in both mice and humans (Webb and Nowowiejski, 1978; Fisher *et al.*, 1981). Therefore, it is possible that the suppressor cell populations induced by MAN are a result of increased PGE_2 production. Suppressor cell induction by PGE_2 has been linked to the immune suppression seen in patients infected with *Coxiella burnetii* (Koster *et al.*, 1985).

Rather than induce specific suppressor cells, PGE_2 may have general inhibitory properties. For example, PGE_2 can inhibit lymphocyte proliferation by suppressing IL-2 production (Nelson *et al.*, 1991). Additionally, Witkin and colleagues noted

reduced proliferative responses by peripheral blood mononuclear in patients with recurrent *Candida* vaginitis that could be reversed by treatment with indomethacin (Witkin *et al.*, 1983; Witkin *et al.*, 1986). Hence, the effects of PGE_2 may be mediated by suppression of T-cell function, rather than the induction of suppressor T-cells.

The effects of PGE_2 may be mediated by immune deviation rather than immune suppression. Excess PGE_2 may lead to preferential activation of the Th2 subsets of T helper cells. PGE_2 increases IL-4 mediated production of IgE and IgG1 (Roper *et al.*, 1990) and promotes a switch to IgE in precommitted B-cells (Roper *et al.*, 1995). Elevated IgE levels have been reported in the serum of patients with high serum PGE_2 (Leung *et al.*, 1988). PGE_2 inhibits the production of IL-2 and IFNγ, but not IL-4 and IL-5 in murine $CD4^+$ cells (Betz and Fox, 1991). In human $CD4^+$ cells, PGE_2 inhibits the production of IL-2 and IFNγ, and enhances IL-4 and IL-5 production (Snijdewint *et al.*, 1993). Finally, PGE_2 is a potent inhibitor of human IL-12 and moderately enhances IL-10 production, possibly by increasing cAMP concentration (van der Pouw Kraan *et al.*, 1995).

Murine models of disease have shown that treatment of mice with indomethacin, an inhibitor of the cyclooxygenase pathway and hence PGE_2 production, is effective in protecting animals from infection (Edwards *et al.*, 1986; Ruebush *et al.*, 1986; Farrell and Kirkpatrick, 1987; Stevens *et al.*, 1992). In two of these studies (Edwards *et al.*, 1986; Stevens *et al.*, 1992) both indomethacin and IFNγ had similar protective effects. Stevens *et al* (1992) showed no synergistic or additive effect when IFNγ and indomethacin were given concurrently. This suggests that both agents function by inhibiting the generation of a Th2 response. These result are consistent with the hypothesis that PGE_2 has its effect through production of a Th2 response rather that a general suppression of cell-mediated immunity.

1.10 THE POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) was first described by Saiki and colleagues in 1985 (Saiki *et al.*, 1985) and has since found application in a range of biomedical research areas. The PCR functions by repeated cycles of denaturation, annealing and extension of target DNA to amplify specific DNA sequences well over a million fold. The initial method (Siaki *et al.*, 1985) used the Klenow fragment of *E. coli* DNA polymerase 1 to amplify DNA. The heat labile nature of this enzyme required the addition of fresh enzyme at the end of each denaturation step. The isolation of a thermostable stable DNA polymerase (Taq polymerase) from a thermophilic bacterium, *Thermus aquaticus*, allowed for the use of PCR without the continuous replenishment of enzyme (Siaki *et al.*, 1988).

Taq polymerase requires a single stranded DNA template to synthesis new DNA. In the first step of the PCR, double stranded DNA (dsDNA) is denatured to form single stranded DNA (ssDNA) (Figure 1.1). Taq polymerase synthesises DNA by adding nucleotides via the 3' hydroxyl group of an existing nucleotide. Consequently, Taq polymerase requires a short sequence of DNA, called a primer, that binds to the template DNA to provide a free 3' hydroxyl group. During the second step, the annealing step, the reaction temperature is reduced to allow primers to anneal to the DNA template. The reaction temperature is then raised to allow Taq polymerase to synthesis DNA by the addition of nucleotides complementary to those of the template, to the free 3' hydroxyl group of the primer (Figure 1.1). In general, the denaturation temperature is ~94°C, the annealing temperature is 55-60°C, depending on the melting temperature (tm) of the primers, and the extension temperature is 70-72°C.

Amplification of target sequence is achieved by the use of two primers. Primers are designed such that one primer is complementary to one of the ssDNA templates and the second primer to the other ssDNA template. Each primer anneals to the 3' end of

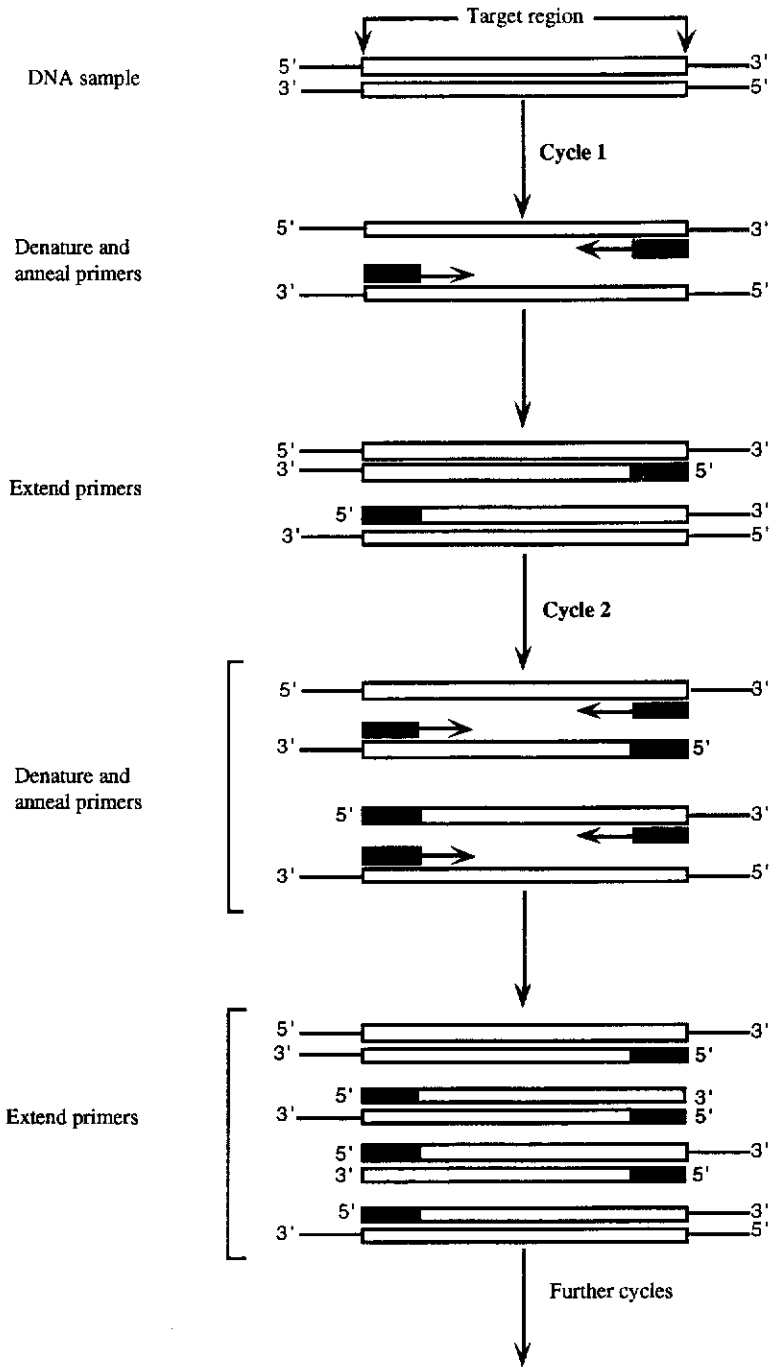

Figure 1.1 Schematic representation of cycle one and two of a PCR. In cycle one the target DNA is heat denatured. Lowered temperature during the annealing step allows for the annealing of primers to specific sites. Raising of the temperature to 70-72°C allows the Taq polymerase to extend the DNA from the primers synthesising a DNA product with a defined 5' end. In cycle two the process is repeated with more product having defined 5' ends, however, now some of the product has both 5' and 3' ends defined by the primer sequence. This process continues exponentially so that following 20-40 cycles most of the product is a defined length, dictated by the distance between the 5' ends of the primer pair.

the respective ssDNA template. The PCR products of the first cycle of amplification therefore have a 5' end that is defined by the 5' phosphate of the primer (Figure 1.1). PCR products from the first round of amplification are then used as a templates for the alternate primer in a second round of amplification. PCR product length from the second round of amplification is therefore circumscribed by the 5' end of the first primer and the 5' end of the second primer (Figure 1.1). The PCR products of a defined length then serve as templates for exponential amplification. The original template DNA remains present and is used as a template throughout the PCR. DNA "copied" from the original template does not have a defined length as only one end is limited by a primer. These products increase in a linear fashion only. Consequently, following a conventional PCR most of the product has a defined length. PCR product length is determined by the distance between the 5' ends of the primer pair.

Since its initial description, many variations on the basic PCR have been described, such as, asymmetric PCR (Gyllensten and Erlich, 1988; Finckh *et al.*, 1991) and inverse PCR (Ochman *et al.*, 1988; Silver and Keerikatte, 1989). One modification of the standard PCR is the reverse transcriptase (RT)-PCR. Taq polymerase can not use mRNA as a template. To circumvent this problem mRNA is used as a template for enzymes called reverse transcriptases that copy mRNA to DNA, this so called copy DNA (cDNA) can then be used for PCR.

1.11 QUANTITATIVE PCR

PCR was first used in a semi-quantitative fashion in 1988 (Chelly *et al.*, 1988) and in a quantitative fashion in 1989 (Becker-André and Hahlbrock, 1989; Wang *et al.*, 1989a). Quantitative PCR has since been used in a range of research areas, from cancer research (Murphy *et al.*, 1990; Luqmani *et al.*, 1992; Bavin *et al.*, 1993), to developmental biology (Watson and Milbrandt, 1990), HIV research (Arrigo and Chen, 1991; Herbein *et al.*, 1994) and immunology (Gilliland *et al.*, 1990; Platzer *et*

al., 1992; Wolf and Cohen, 1992; Wesselingh *et al.*, 1994). However, quantitative PCR has been criticised for a number of reasons, such as lack of exponential amplification in later stages of the PCR and competition between target sequences for reagents during amplification. To answer these criticisms several variations of quantitative PCR have been designed (Becker-André and Hahlbrock, 1989; Wang *et al.*, 1989a; Gilliland *et al.*, 1990; Luqmani *et al.*, 1992). However, there remains three main factors, the plateau effect, tube effects and template quality, that affect PCR quantitation to varying degrees.

1.11.1 Factors affecting PCR quantitation

1.11.1.1 The Plateau effect

The plateau effect is arguably the greatest impediment to the development of quantitative PCR methodologies. In the initial cycles of a PCR the amplification of template is exponential. However, as the reaction proceeds the dynamics of the reaction change and the amplification efficiency drops. The loss of exponential amplification is due to a range of factors, such as, substrate saturation of enzyme, product inhibition of enzyme, incomplete strand separation, and product strand reannealing (Larzul *et al.*, 1988). The cycle number at which the reaction will plateau cannot be predicted as it depends on the template concentration and the average amplification efficiency and must, therefore, be determined empirically. Only during the exponential or logarithmic phase of the PCR is the amount of starting template related to final PCR product yield. Therefore, determination of the logarithmic range of amplification is essential for most forms of quantitative PCR.

1.11.1.2 The tube effect.

For reasons that are not clear, a single PCR "master mix" (ie a bulk mix of reagents) aliquoted into separate tubes and subjected to the same amplification regime can result in variations in the final yield of product. Various explanations have been proposed for this anomaly including, sampling errors (small errors would produce large effects due to the sensitivity of the assay), temporary changes in local substrate concentrations, tube "edge effects" and variations in temperature across the heating block. Presumably this phenomenon is due to a combination of some or all of these factors and would change with different operators, equipment and laboratories. To circumvent these problems many quantitative PCR regimes make use of an internal standard which is co-amplified with the target sequence (Chelly *et al.*, 1988; Becker-André and Hahlbrock, 1989; Wang *et al.*, 1989a; Gilliland *et al.*, 1990; Luqmani *et al.*, 1992). Comparison of the target to the internal standard counteracts inter tube variation.

1.11.1.3 Template Quality

Template quality needs to be considered when attempting to determine concentration of template. In general, no specific precautions need to be taken to preserve DNA integrity. However, the chemically reactive structure of RNA and the plethora of highly stable exogenous and endogenous RNases makes RNA samples highly susceptible to degradation (Sambrook *et al.*, 1989). The mRNA integrity can be determined by electrophoresis and EtBr staining (Sambrook *et al.*, 1989) or by northern blotting and probing for an abundant message such as that for β-actin. However, these methodologies require substantial amounts of RNA and the power of the PCR technique relies on the ability to use small samples.

Many quantitative PCR methodologies rely on endogenous standard such as β-actin mRNA to control for RNA degradation. Since the endogenous standard mRNA is subject to the same conditions as the target mRNA a ratio of target to standard negates the problems of mRNA degradation (Chelly *et al.*, 1988; Luqmani *et al.*, 1992; Iizawa *et al.*, 1993). Copy RNA controls are used in other techniques to control for mRNA degradation (Becker-André and Hahlbrock, 1989; Wang *et al.*, 1989a; Babu, 1993). Copy RNA (cRNA) is mRNA that has been transcribed from plasmid DNA. The cRNA and sample RNA are then subjected to the same RT reaction. Consequently, the cRNA, when used as a standard in a subsequent SQ-PCR, controls for RNA degradation (Becker-André and Hahlbrock, 1989; Wang *et al.*, 1989a; Gilliland *et al.*, 1990).

1.11.2 Types of Quantitative PCR

Several methods for quantifying DNA or cDNA, that attempt to address problems such as the plateau effect and the 'tube effect', have been devised. Although most have their own intrinsic limitations, with the use of appropriate controls, template DNA can be quantified or at least semi-quantified. The term semi-quantitative implies that differences between samples can be detected. Exact values can not be given for each sample, rather the relative amounts of product are compared (Chelly *et al.*, 1988; Luqmani *et al.*, 1992; Iizawa *et al.*, 1993).

1.11.2.1 The 'Endogenous Standard' assay

This method utilises an endogenous mRNA sequence, such β-actin mRNA, that is expressed at relatively constant levels in all tissues (Chelly *et al.*, 1988; Luqmani *et al.*, 1992; Babu, 1993; Iizawa *et al.*, 1993). The PCR is performed with primers for both the nucleic acid target sequence of interest and the endogenous standard sequence in

the same reaction tube. The use of both primer sets in the same tube seeks to by pass any "tube effects".

In the exponential phase of the PCR, the yield of product, plotted in a log scale, against cycle number produces a straight line. The gradient of the line produced represents the amplification efficiency of the reaction. For accurate results the gradient of the standard curve for the target and endogenous standard should be similar (Luqmani *et al.*, 1992). Test samples can then be normalised to the endogenous standard and compared to other samples corrected in the same fashion (Chelly *et al.*, 1988). As each sample is calibrated with an internal control, differences in RT efficiency and RNA degradation between samples is negated.

The endogenous standard assay is the least laborious of the quantitative PCR methods. As the standard is already present in each sample it is useful when a large number of samples need to be assayed. However, this technique has a number of technical problems, such as differences in the amplification efficiencies between target and standard sequence.

The yield of product in a PCR is proportional to starting DNA concentration according to the formula $N=N_0(1+eff)^n$, where N is the product yield, N_0 is the starting concentration and eff is the efficiency of the amplification (Wang *et al.*, 1989a). A small change in amplification efficiency will have a dramatic effect on the product yield, which is exaggerated as cycle number increases. Differences in amplification efficiency can be due to a number of variables, such as template concentration, template composition and length and tm of primer and template (Larzul *et al.*, 1988). Differences in amplification efficiency of up to 10^5 have been reported between different primer/template sets (Wang *et al.*, 1989a). A fundamental requirement in the endogenous standard technique is for all the primers to have similar amplification

efficiencies over the appropriate concentration range used and to avoid entry into the plateau phase by minimising cycle number.

A potential problem unique to the endogenous standard technique is that the standard sequence may not be produced at a consistent level by all types of cells. As the standard is used to estimate the concentration of the test sequence any variation in standard concentration will bias the results. Hence, if the standard sequence is highly expressed, the target sequence will be under estimated. In the converse, if the concentration of the standard sequence is low, the target sequence will be overestimated. However, comparisons of different house keeping genes have indicated that this may be an over stated concern (Platzer *et al.*, 1992).

Potentially the greatest problem with the endogenous standard technique, given equal amplification efficiencies, is that many of the test sequences are expressed at considerably lower levels than the control (Babu, 1993). Hence, detection of test sample is hindered by the concentration of the standard which may compete more effectively for the PCR reaction components. This problem can be overcome by the use of control sequences which are at similar concentrations to the test sequence (Chelly *et al.*, 1988).

1.11.2.2 Synthetic internal standards

Synthetic internal standards can be used as a means of controlling variation due to factors such as "tube effects". In this assay, a constant amount of synthetic internal standard is added to each sample prior to PCR. Standards are designed so that amplification efficiencies of standard and test are identical (Wang *et al.*, 1989a). Serial dilutions of target and internal standards are subjected to PCR amplification. If target and standards have similar amplification efficiencies a standard curve of each will yield parallel lines. The concentration of test sequence is determined by comparison to the

synthetic internal control (Wang *et al.*, 1989a). Synthetic internal standards are differentiated either on size or by the engineering of a new restriction endonuclease (RE) site (Wang *et al.*, 1989a; Hart *et al.*, 1990; Lin *et al.*, 1991).

The amount of standard needs to be determined empirically as does the number of cycles (Wang *et al.*, 1989a). This method involves substantial optimisation but does allow for determination of absolute numbers of specific mRNA molecules in each sample. RT efficiency can be controlled for by the addition of cRNA standards to the RNA prior to the RT step. This method has been used to calculate the absolute number of target mRNA species per cell (Wang *et al.*, 1989a; Funk and FitzGerald, 1991).

The major problem with the internal standard method is the narrow concentration range that can be used for each internal standard. If the internal standard is in excess then competition between standard and target sequence for primers and other reaction components can bias the results (Babu, 1993). Conversely, if the standard is too low it is not possible to extrapolate results from the standard curve to the test curve, or the target sequence will compete more effectively for the PCR reaction components than the standard sequence. Consequently, the system needs extensive optimisation for each new mRNA target to ensure an appropriate ratio of target to standard, this can only be determined empirically. Also, variability between target mRNA levels in different samples can result in the need for re-calibration of the system with each new sample or sample type (Funk and FitzGerald, 1991; Babu, 1993).

1.11.2.3 Competitive PCR.

In 1989 Becker-Andre and Hahlbrock described a competitive quantitative PCR that relied on competition between internal standard and target sequence to define the concentration of the target. As in the synthetic internal standard assay, both target

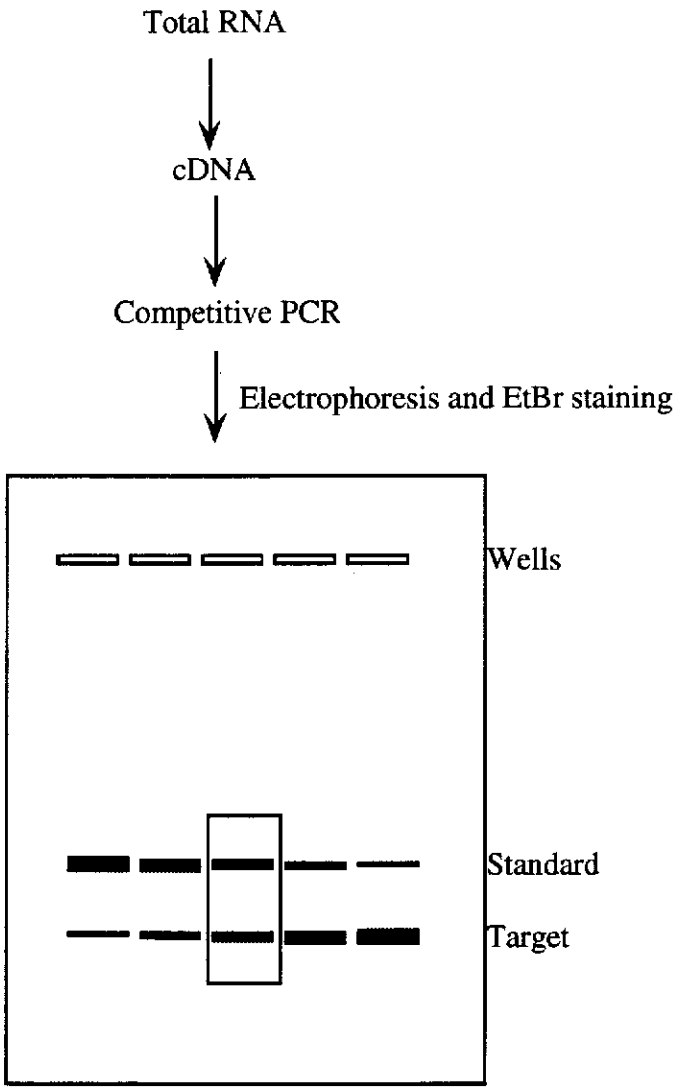

Figure 1.2: Competitive quantitative PCR. Serial dilutions of standard DNA is added to a constant amount of target. During PCR the standard competes with the target sequence for reaction components such as primers and dNTPs. Following PCR the products are separated on an agarose gel and stained with EtBr. The boxed bands represent the concentration at which competitive standard and target sequences are at equivalence and equates to the starting concentration of the target DNA.

sequence and standard use the same primer. The addition of a second sequence within the PCR that uses the same primer as the target sequence sets up competition for primers and other reaction components with the target sequence. Assuming equal amplification efficiencies, the degree of competition will be determined by the amount of competitor in relation to target sequence. When the concentration of competitor is low the competition will be low and PCR product for the target sequence will dominate. The converse would occur when the concentration of competitor is high. Between these two extremes there will be a point of equivalence, where the ratio of target to competitor is equal. Target concentration can be determined by the concentration of the standard at which the point of equivalence is reached (seen as the boxed products in Figure 1.2).

The competitive sequences used in the competitive PCR technique are similar to those used in the internal standard assay (Gilliland *et al.*, 1990; Platzer *et al.*, 1994). Plasmids have been constructed with multiple competitive sequences so that one plasmid can be used as a control for many different gene products (Platzer *et al.*, 1994). This approach has also been adopted in the synthetic internal standard approach (Wang *et al.*, 1989a; Funk and FitzGerald, 1991).

As with the internal standard method, cRNA can be used to control for RT efficiency so that absolute numbers of mRNA template can be determined (Huang *et al.*, 1994). The concentration range of competitive DNA added must be broad enough to cover the point of equivalence with the test sequence. Since this is unknown, a broad dilution of competitive DNA is used (Gilliland *et al.*, 1990) which is narrowed down when the area of equivalence is determined. As competitive PCR assay is based on competition, the reaction can be allowed to enter the plateau phase without affecting quantitation.

1.12 CAVEATS OF mRNA MEASUREMENT

The advent of RT-PCR allowed for qualitative and quantitative determination of cytokine gene expression in circulating leukocytes and in the individual tissues in normal and disease states (Luqmani *et al.*, 1992; Platzer *et al.*, 1992; Wolf and Cohen, 1992; Kramnick *et al.*, 1993; Murphy *et al.*, 1993; Delassus *et al.*, 1994; Fox *et al.*, 1994; Herbein *et al.*, 1994; Huang *et al.*, 1994; Platzer *et al.*, 1994; Ramakrishnan *et al.*, 1994). However, whilst the RT-PCR can be used to quantitate mRNA, the amount of mRNA may not necessarily reflect the amount of translated protein. However, there is substantial evidence to suggests that levels of mRNA are a reliable indicator of protein synthesis. The major mechanism of cytokine protein regulation is transcriptional (Howard *et al.*, 1993). Additionally, many cytokine mRNA species, such as IL-1, IL-2, GM-CSF, granulocyte-colony stimulating factor (G-CSF), IFNγ and TNF have AUUA rich sequences suggesting they are post-transcriptionally regulated by susceptibility or resistance to RNA degradation (Howard *et al.*, 1993). The combination of transcriptional regulation and post-transcriptional regulation by susceptibility to RNases allows for the extrapolation of mRNA levels to protein levels. Consequently, the level of cellular mRNA should be an accurate reflection of the level of secreted protein. Indeed, at least in T-cells, this seems to be the case (Kelso, 1993). However, caution needs to be maintained, as cytokines such as, IL-1 and TNFα, can be regulated at the translation and post-translational levels (Elias *et al.*, 1989; Han and Beutler, 1990; Hazuda *et al.*, 1990).

AIMS OF THE RESEARCH

To characterise the tissue histology and tissue distribution patterns of *C. albicans* during systemic murine candidiasis.

To develop a reliable, reproducible and sensitive SQ-RT-PCR for the quantitation of *in vivo* cytokine gene transcription.

To use this technique to determine the *in vivo* pattern of tissue specific cytokine gene expression during systemic candidiasis.

To determine if cytokine gene expression patterns vary between resistant BALB/c and sensitive CBA/CaH mice during primary systemic candidiasis.

To determine if differences in tissue distribution of *C. albicans* in infected mice is matched by differences in tissue responses to infection.

To determine if cytokine mRNA expression patterns during secondary systemic candidiasis, are different to those during primary systemic candidiasis.

To determine if cytokine gene expression patterns vary between resistant BALB/c and sensitive CBA/CaH mice during secondary systemic candidiasis.

CHAPTER TWO

MATERIALS AND METHODS

MATERIALS

2.1 GENERAL MATERIALS

2.1.1 Mice

Specific pathogen-free BALB/c and CBA/CaH mice were obtained from the Animal Resource Centre (ARC), Murdoch University, Perth, Western Australia. Mice are subjected to routine genetic and microbiological monitoring (by ARC) and do not carry *C. albicans* in the gut. Mice used in this study were all females, between six to eight weeks of age. Mice were kept under NH&MRC guide lines on a 12 hour light dark cycle. Food and water were supplied *ad libitum*.

2.1.2 *Candida albicans*

Candida albicans strain KEMH5 was obtained from Dr K. Ott, Department of Pathology, King Edward Memorial Hospital for Women, Perth Western Australia. The strain was taken from a patient with vulvo-vaginal candidiasis. Identification was performed by a standard germ tube test (Finegold and Baron, 1986). *C. albicans* strain KEMH5 was stored as a glycerol stock at -20°C.

2.2 ANTIBODIES USED IN IMMUNOHISTOCHEMISTRY

Antibodies used in immunohistochemistry are shown in Table 2.1. The table shows the name of the hybridoma, the Ag, cell distribution of the Ag and original reference. Ly-1, M5/114, secondary Ab (biotinylated sheep anti-rat) and horse radish peroxidase conjugated streptavidin (SA-HRP) were obtained commercially (Boehringer Mannheim, Germany or Dako, U.S.A). Ser-4 was obtained from Dr P. Holt, WA Research Institute for Child Health, Princess Margaret Hospital for Children, Perth, Western Australia. All other hybridomas were obtained form Dr P Price, Department of Microbiology, University of Western Australia, Nedlands, Western Australia.

Antibody (isotype)	Antigen	Cell distribution	Reference
GK1.5 (IgG2a)	CD4	T-cells and dendritic cells	(Dialynas *et al.*, 1983a; Dialynas *et al.*, 1983b)
53-6.72 (IgG2a)	CD8a/Ly-2	T-cells	(Ledbetter and Herzenberg, 1979)
KT3.2 (IgG2a)	CD3δ	T-cells	(Tomonari and Lovering, 1988)
M5/114.15.2 (IgG2a)*	H-2 Ia $I\text{-}A^{b,d,q}$ and $I\text{-}E^{dk}$	Various	(Bhattacharya *et al.*, 1981)
M1/70 (IgG2a)	Mac-1; CD11b	macrophages, monocytes, granulocytes, B-cell subsets and NK	(Springer *et al.*, 1979)
F4/80 (IgG2b)		Predominantly Macrophages, also on eosinophils and epithelial like cells in BM stromal culture	(Austyn and Gordon, 1981)
Ser-4 (IgG2a)	SER; siaolo-adhesin	Macrophage subset	(Crocker and Gordon, 1989)
53-7.3 (IgG2a)	CD5/Ly-1	T-cells, B-cell subsets	(Ledbetter and Herzenberg, 1979)
Sheep anti rat (Biotinylated)	Rat immunoglobulin		

Table 2.1 Antibodies used in Immunohistochemistry.
* M5/114 Ab was used predominantly only on BALB/c mice as the Ab reacts with $I\text{-}A^{d}$ on BALB/c mice but not the $I\text{-}A^{k}$ on CBA mice. While the Ab reacts with $I\text{-}E^{k}$, the expression of $I\text{-}E^{k}$ is low compared to $I\text{-}A^{d}$ (Bhattacharya *et al.*, 1981).

2.3 MOLECULAR BIOLOGY MATERIALS

2.3.1 PCR Primers

Primers were produced on a 381A ABI DNA synthesiser or were obtained commercially from Integrated DNA technologies, INC. (U.S.A). All primers, except those obtained commercially, were assessed for purity by end labelling and electrophoresis (Sambrook *et al.*, 1989). Sequences of the 5' and 3' primers were as follows;

IL-1α 5' primer CTGCAGTCCATAACCCATGA
IL-1α 3' primer GAGGTCGGTCTCACTACCTG
Amplified DNA size from cDNA: 375bp
Reference cDNA: (Lomedico *et al.*, 1984)

IL-2 5' primer GTGCTCCTTGTCAACAGCGC
IL-2 3' primer GAGCCTTATGTGTTGTAAGC
Amplified DNA size from cDNA: 499bp
Reference, cDNA: (Kashima *et al.*, 1985)

IL-3 5' primer AATCAGTGGCCGGGATACCC
IL-3 3' primer CGAAAGTCATCCAGATCTCG
Amplified DNA size from cDNA: 299bp
Reference, cDNA: (Fung *et al.*, 1984)

IL-4 5' primer TCTTTCTCGAATGTACCAGG
IL-4 3' primer CATGGTGGCTCAGTACTAGG
Amplified DNA size from cDNA: 400bp
Reference, cDNA: (Lee *et al.*, 1986)

IFNγ 5' primer CATGAAAATCCTGCAGAGCC
IFNγ 3' primer GGACAATCTCTTCCCCACCC
Amplified DNA size from cDNA: 310bp
Reference, cDNA: (Gray and Goeddel, 1983)

TNFα 5' primer CCTGTAGCCCACGTCGTAGC
TNFα 3' primer TTGACCTCAGCGCTGAGTTC
Amplified DNA size from cDNA: 373bp
Reference, cDNA: (Fransen *et al.*, 1985)

M-CSF 5' primer AGCCACATGATTGGGAATGG
M-CSF 3' primer ATGGAAAGTTCGGACACAGG
Amplified DNA size from cDNA: 299bp
Reference, cDNA: (DeLamarter *et al.*, 1987)

G-CSF 5' primer AGGAGCTGGTGTTGCTGGGC
G-CSF 3' primer GAAGTGAAGGCTGGCATGGC
Amplified DNA size from cDNA: 291bp
Reference, cDNA: (Tsuchiya *et al.*, 1986)

GM-CSF 5' primer CCTGAGGAGGATGTGGCTGC
GM-CSF 3' primer GGCTGTCATGTTCAAGGCGC
Amplified DNA size from cDNA: 294bp
Reference, cDNA: (Gough *et al.*, 1985)

β-actin 5' primer CTGAAGTACCCCATTGAACATGGC
β-actin 3' primer CAGAGCAGTAATCTCCTTCTGCAT
Amplified DNA size from cDNA: 762bp
Reference, cDNA: (Alonso *et al.*, 1986)

2.3.2 Positive control DNA

Plasmids containing the cDNA inserts for the cytokines IL-2 (pcD Mo IL-2), IL-3 (pcD Mo IL-3), IL-4 (pcD Mo IL-4), IFNγ (pcD Mo IFNγ), GM-CSF (pcD Mo GM-CSF) and G-CSF (pMG2) were used as a source of positive control DNA for the development of the SQ-PCR and as standards for later analysis of cDNA samples. Plasmids for IL-2, IL-3, IL-4, IFNγ and GM-CSF were provided by Alistair Rahmsay, John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory. Plasmid, pMG2, was a gift from Alison Smith, Walter and Elisa Hall Institute for Medical Research, Melbourne, Victoria.

2.4 BUFFERS AND SOLUTIONS

All buffers and stock solutions not included in this section were prepared as described in Sambrook *et al.* (1989). All chemicals and enzymes of a suitable grade were purchased commercially and were not further purified. All solutions were prepared using distilled deionised water (Millipore Hi-pure water, hpH_2O). When appropriate, solutions were sterilised either by autoclaving at 121°C, 103kPa for 15 minutes, or by filtration through a 0.22µm filter.

Restriction endonucleases were purchased from Boehringer-Mannheim, Pharmacia, Promega or Toyobo. Taq DNA polymerase was purchased from Cetus. RNAsin and AMV reverse transcriptase was purchased from Promega.

2.4.1 Media

Media was sterilised by autoclaving at 121°C, 103 kPa for 15 minutes. Antibacterial agents were prepared in hpH_2O and sterilised by filtration through a 0.22µm filter. Filtered antibacterial agents were added prior to use of media broth or to agar broth (when at 55°C) prior to pouring plates

2.4.1.1 Sabourauds Agar (SAB) + Chloramphenicol

Peptone	1%
Agar	1.5%
Glucose	4%
Chloramphenicol	50 µg/ml

2.4.1.2 Terrific broth

Bactotryptone	1.2%
Bactoyeast	2.4%
Glycerol	0.4%
K_2HPO_4	71 mM
KH_2PO_4	17 mM
Ampicillin	100 µg/ml

The broth was prepared according to the method detailed in Sambrook, *et al.* (1989).

2.4.1.3 Yeast extract Peptone-Dextrose broth (YEPD)

Yeast extract	1%
Peptone	2%
Glucose	2%

YEPD agar was prepared by adding agar (Difco) to 1.5%

2.4.2 Histological stains and solutions

2.4.2.1 Eosin

Eosin	6.2% (w/v)
Alcohol	80%
Phloxine	0.1% (w/v)
Acetic acid	1%

Stock Eosin was made by dissolving 25g of water soluble eosin in 4 litres of 80% alcohol containing 1g of phloxine and 40ml of glacial acetic acid.

2.4.2.2 Harris Haematoxylin (Hx)

Haematoxylin (Hx)	0.46%
Alcohol	4.59%
Ammonium Alum	386.9 mM
Mercuric Oxide Red	10.6 mM
Acetic acid	3.67%

Hx was prepared by dissolving 0.5g of Hx in 10ml of ethanol. Ammonium Alum (10g) was then dissolved in 100ml of hot hpH_2O and the Hx solution added. The solution was boiled and 0.25g of HgO added and cooled rapidly. Glacial acetic acid (4ml) was added and the solution was filtered before use.

2.4.2.3 Periodic Acid Shiffs (PAS)

PAS reagents, 5% periodic acid and Shifts reagent, were prepared according to the method described in Bancroft and Stevens (1990).

2.4.3 Immunohistochemistry

2.4.3.1 Diaminobenzidine (DAB)

DAB stocks were made by dissolving 12mg of DAB in 10ml of PBS (2.4.3.2). DAB was stored at -20°C in 200µl aliquots. For use, 200µl of stock DAB was diluted with 800µl of PBS and added to 1ml of 0.02% H_2O_2. Diluted DAB was used immediately.

2.4.3.2 PBS (pH7.2)

NaCl	150mM
Na_2HPO_4	16mM
$NaH_2PO_4.2H_2O$	4mM

PBS was made in hpH_2O and the pH was adjusted to 7.2. PBS was sterilised by autoclaving and stored at RT.

2.4.4 Standard molecular biology reagents

2.4.4.1 DNA loading buffer (5x)

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	50%
EDTA, pH8.0	1mM

The buffer was made in hpH_2O.

2.4.4.2 PCR buffer (10x)

KCl	500mM
Tris-HCl (pH8.3)	100mM
$MgCl_2$	2mM
Gelatin	0.1% w/v

PCR buffer was made in hpH_2O, sterilised by autoclaving and stored at RT.

2.4.4.3 TBE (5x)

Tris base	0.45mM
Boric acid	0.45mM
EDTA pH8.0	10mM

TBE was made up in hpH_2O and stored at RT.

2.4.4.4 TE buffer, pH 8.0

Tris-HCl, pH 8.0	10mM
EDTA, pH 8.0	1 mM

TE was made up in hpH_2O, sterilised by autoclaving prior to use and stored at RT.

2.4.4.5 Tris-Acetate Electrophoresis Buffer, pH 8.0 (TAE), (50x)

Tris-base	2 M
EDTA	0.1 M
Acetic acid	5.71%

The solution was diluted 1:50 in hpH_2O and used for electrophoresis.

2.4.5 Total RNA extraction

2.4.5.1 Preparation of glassware

All glassware used for the extraction of RNA or the storage of RNA solutions was washed in a solution of 0.1% diethylpyrocarbonate (DEPC) in hpH_2O. Glassware was then dried in an oven at 180°C for 2-3 hours.

2.4.5.2 DEPC treated solutions for RNA techniques

DEPC was allowed to equilibrate in hpH_2O or required solutions for 48 hours. DEPC was removed by autoclaving twice at 121°C, 103 kPa for 15 minutes. Tris-base solutions were prepared in DEPC treated hpH_2O and the pH adjusted accordingly.

2.4.5.3 Ethidium Bromide

Ethidium bromide (EtBr) for RNA electrophoresis was taken from stock 10mg/ml (made in DEPC treated hpH_2O) solution and diluted 1:20 in DEPC treated hpH_2O to 0.5mg/ml. The EtBr solution was stored at 4°C in a foil wrapped bottle.

2.4.5.4 Formaldehyde gel-loading buffer

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	50%
EDTA, pH8.0	1mM

The buffer was made in DEPC treated hpH_2O.

2.4.5.5 Formaldehyde gel running buffer (5x)

Morpholinopropane sulphonic acid (MOPS)	0.1M
Sodium acetate, pH 7.5	40mM
EDTA, pH8.0	5mM

The formaldehyde gel running buffer was made by dissolving MOPS in 50mM DEPC treated sodium acetate. The pH was adjusted to 7.0 and 0.5M DEPC treated EDTA added to 5mM. The formaldehyde gel running buffer was stored at -20°C.

2.4.5.6 Guanidinium solution

Guanidinium thiocyanate	4M
Sodium citrate pH7.0	25mM
N-lauryl sarcosine (sarcosyl)	0.5%
Dithiothreitol (DTT)	0.1mM

The guanidinium solution was prepared in DEPC treated hpH_2O and stored in the dark at RT. The solution was stable for three months. The DTT was added just prior to use from a frozen 10mM stock. The guanidinium solution, with DTT added, was stable for up to one month when stored in the dark at RT.

2.4.5.7 Phenol was prepared by melting solid phenol at 55°C and equilibrating in 0.1M Tris containing 0.01% 8-hydroxyquinoline. Phenol was then equilibrated a further two times in DEPC treated hpH_2O (2.4.5.2) and stored under fresh DEPC treated hpH_2O for up to one month. Alternatively, phenol was stored indefinitely at -20°C until needed, when at RT, the phenol was used within one month

2.5 GENERAL METHODS

2.5.1 *Candida albicans* culture conditions

For a working culture of *C. albicans* (2.1.2), glycerol stocks were streaked onto SAB plates (2.4.1.1) and incubated at 30°C for 48 hours. Short term storage of *C. albicans* was on SAB plates at 4°C. For use *C. albicans* were grown in YEPD broth (2.4.1.3) o/n at 30°C in a shaking water bath.

2.5.2 Inoculation of mice to induce systemic candidiasis

Over night cultures (2.5.1) of *C. albicans* KEMH5 were pelleted by centrifugation at 2 000g for 10 minutes and washed twice in 10mls of 0.9% pyrogen free sterile saline (Delta West). Pelleted yeast cells were resuspended in 10ml of 0.9% pyrogen free sterile saline. Blastospores were counted in an improved Neubauer counting chamber. *C. albicans* KEMH5 was diluted in 0.9% pyrogen free sterile saline to the appropriate concentration.

BALB/c and CBA mice were inoculated by intravenous (*iv*) injection into the tail vein with 200µl of *C. albicans* inoculum. Control mice were inoculated with 200µl of 0.9% pyrogen free sterile saline.

2.5.3 Quantitative yeast culture

Mice were sacrificed by cervical dislocation and target organs removed for homogenisation. Six tissues were removed from each mouse; the kidneys, brain, spleen, liver, lungs, and the heart. Infection was assessed in each tissue by homogenisation of the tissue (100mg) in 2ml sterile saline in a glass/teflon

homogeniser. The resultant homogenate (50µl) was platted onto SAB plates containing chloramphenicol (2.4.1.1). Serial 1/10 dilutions of tissue homogenate were made prior to plating, if required. Dilutions were routinely applied to kidney and brain homogenates and to homogenates of other tissues following a $1x10^6$ inoculation of *C. albicans*. Colonies of *C. albicans* were counted after 48 hours. One colony was equivalent to 400 colony forming units/gram (cfu/g) of tissue. Results were expressed as $\log_{10}$ cfu/g tissue.

2.5.4 Indomethacin treatment

Stock solutions of indomethacin (Sigma) were prepared by dissolving in 100% ethanol at 25 or 50mg/ml. Dilutions of indomethacin were then made in sterile PBS (2.4.3.2) for *iv* injection or in normal drinking water for oral administration. Indomethacin was administered *iv* by tail vein injection at the appropriate concentration in 200µl volumes. Control mice received equivalent injections of ethanol in PBS. Indomethacin was administered orally by diluting indomethacin in normal drinking water. Control mice were treated with equivalent concentrations of ethanol in the drinking water. Drinking water was changed every 2-3 days.

2.6 HISTOLOGY

2.6.1 Tissue processing and embedding

Mouse tissue was fixed in 10% buffered formal saline (Bancroft and Stevens, 1990) for a period of 3-7 days. Tissue processing was performed on an automated Shandon Hypercenter using a standard protocol. All steps were carried out under vacuum at RT. Following processing, the tissues were embedded in paraffin wax blocks, using plastic moulds (Miles Laboratories Pty Ltd). Processed tissues were embedded either as one tissue type per block or as a composite blocks

2.6.2 Sectioning of paraffin wax embedded tissues

Paraffin wax embedded tissues were cooled to 4°C for 30-60 minutes on ice. Tissue blocks were then trimmed in a Spencer 820 microtome placed back on ice and then sectioned to a thickness of 5μm. Tissue sections were dried and stored at RT until needed.

2.6.3 Staining

2.6.3.1 Haematoxylin and Eosin (H&E)

Paraffin wax embedded sections (2.6.2) were hydrated following standard techniques (Bancroft and Stevens, 1990) and stained with H&E (2.4.2.1, 2.4.2.2) by standard techniques (Bancroft and Stevens, 1990).

2.6.3.2 PAS staining

Paraffin wax embedded sections (2.6.2) were hydrated following standard techniques (Bancroft and Stevens, 1990) and stained by the PAS (2.4.2.3) method using standard techniques (Bancroft and Stevens, 1990).

2.7 IMMUNOHISTOLOGY

2.7.1 Frozen tissue sectioning

Tissues were removed from mice following cervical dislocation and placed into moulds containing OCT (Miles Ltd). Tissues were frozen by placing over liquid nitrogen and were stored at -80°C until needed. Tissues were sectioned in a cryostat (Reichert-Jung Cryo-Cut II) at 9μm. Multiple sections, sufficient for the panel of Ab used (Table 2.1), were taken from each tissue composite block. Tissues were surveyed by taking a series of sections 90μm apart. Sections were air dried at RT and stored at 4°C for no more than 5 days before staining. At least two series of sections, 90μm apart, were stained (2.7.2) by a panel of mAb (Table 2.1).

2.7.2 Immunohistology

Frozen sections (2.7.1) were fixed for 15 minutes in 100% ethanol at 4°C, then washed three times in PBS (2.4.3.2). Sections were incubated in 1% H_2O_2 for 10 minutes at RT to remove endogenous peroxidase activity. In some cases the 1% H_2O_2 treatment was repeated up to 3 times to remove endogenous peroxidase activity from the kidney. Sections were washed three times in PBS. Sections were incubated with primary Ab (section 2.2, Table 2.1) diluted PBS plus 2% FCS (titrations had been performed previously to the determine optimal dilution for each Ab) for 60 minutes at RT. Sections were washed three times in PBS and incubated with secondary Ab (section 2.2, Table 2.1) diluted in PBS plus 2% FCS for 30 minutes at RT. Sections were washed three times in PBS and incubated with SA-HRP diluted in PBS plus 2% FCS for 30 minutes at RT. Sections were washed three times in PBS and the chromogen, DAB (2.4.3.1), was added. The colour was allowed to develop for 10 minutes at RT and the sections were again washed in PBS. Sections were lightly

stained in Hx (2.4.2.2), washed in water and allowed to air dry prior to mounting in DPX (BDH).

2.8 MOLECULAR BIOLOGY METHODS

2.8.1 Acrylamide Gel Electrophoresis

Acrylamide gel electrophoresis was used to separate PCR products for both semi-quantitative and conventional PCR. Acrylamide gels used were 5% acrylamide and 0.17% N, N'-methylenebisacrylamide. Gels were electrophoresed in 1 x TBE (2.4.4.3) using a Bio-Rad Power Supply 200/2.0, and Mini-Protean II (Bio-Rad) or Protean II (Bio-Rad) gel electrophoresis tanks. Following conventional PCR product electrophoresis, gels were stained in EtBr (0.5mg/ml) and photographed (Polaroid CU-5 land camera) for 10 seconds under a UV light using 667 black and white film. Following electrophoresis of radiolabelled PCR product, gels were dried (Bio-rad gel drier 583) and subjected to autoradiography (2.8.3)

2.8.2 Agarose gel electrophoresis (AGE)

The equipment used for AGE consisted of the Bio-Rad Power Supply 200/2.0, a Bio-Rad "Mini-sub" or "Wide mini-sub" Electrophoresis Cell. The appropriate amount of agarose (DNA grade) was dissolved in 1 x TAE buffer (2.4.4.5) by gentle heating. Molten agarose was cooled to 55°C before pouring 20-30 ml onto a 7 x 10 cm perspex tray for the "Mini-Sub", or 50-70 ml onto a 15 x 10 cm perspex tray for the "Wide Mini-Sub", with a well forming comb positioned 1 cm from one end. The gel was allowed to set at RT for 20-30 minutes before the comb was removed, and the gel placed into the electrophoresis chamber containing 1 x TAE buffer. All DNA samples were prepared for electrophoresis by the addition of 5 x DNA loading buffer (2.4.4.1).

The samples were electrophoresed at 70 Volts for 1.5 - 2 hours at RT. The gels were then stained in EtBr solution (0.5mg/ml) for 10-15 minutes. Following electrophoresis the gel was photographed for 10 seconds under a UV light using 667 black and white film.

2.8.3 Autoradiography

Autoradiography was carried out in 35 x 43 cm autoradiography cassettes (Dupont) with single or dual intensifying screens (Dupont cornex lightning plus IH) at -80°C. Exposure time (usually 3-24 hours) was determined by the level of radiation detected by a hand held β-counter.

The film (X-OMAT AR or X-OMAT AO) was developed for 2 minutes in Kodak film developer (1:5 dilution of stock developer), washed in water, then fixed for 2 minutes in Kodak film fixer (1:5 dilution of stock fixer). The film was then washed in water, rinsed in 70% ethanol and allowed to air dry.

2.8.4 Fractionation of, and purification of, cDNA from control plasmids

In some experiments the cDNA inserts rather than complete plasmid (2.3.2) was used as controls for SQ-PCR. In these instances plasmid DNA (2.8.5) was subject to restriction endonuclease digestion. The cDNA was then separated from vector DNA by AGE (2.8.2). Insert cDNA was excised from the agarose gel and purified by the "freeze squeeze" method. A small hole was made in the bottom of a 750µl microfuge tube which was then plugged with a small piece of siliconised glass wool. The agarose gel slice containing the cDNA was placed into the 750µl microfuge which was then snap frozen in liquid nitrogen. This was placed into a 1.5ml microfuge tube (with the lid removed) and centrifuged at 13 000g for 20 minutes. The eluate containing the purified insert was then transferred to a fresh tube and stored at -20°C until needed.

2.8.5 Plasmid purification for PCR standards

E. coli containing the desired plasmid (2.3.2) was taken as a scraping from glycerol stock (-80°C) and inoculated into 10ml of terrific broth, supplemented with ampicillin to a final concentration of 100µg/ml (2.4.1.2). Following o/n incubation at 37°C, the 10ml culture was added to 500ml of terrific broth containing ampicillin (100µg/ml) in a 1 litre flask. This was incubated o/n at 37°C on an orbital shaker (Gyrotory water bath model G76, Brunswick scientific Co. Inc.). *E. coli* cultures were pelleted in two 250ml polypropylene tubes (Nalgene) in a Beckman 'midrange' centrifuge (J2-21M induction drive centrifuge) using a JA-14 rotor, at 10 000g for 3 minutes. Cell pellets were washed by resuspending them in 10 ml sterile TE (2.4.4.4) and centrifuging at 10 000g for 3 minutes. Plasmids were purified by the caesium chloride (CsCl) gradient method according to Sambrook *et al.* (1989). Plasmid concentration was estimated by spectrophotometric measurement (Sambrook *et al.*, 1989).

2.8.6 Polymerase chain reaction

Polymerase chain reactions were carried out in a final volume of 30µl. Concentration of reaction components were 1 x PCR buffer (2.4.4.2), 0.2µM of each sense and anti-sense primer (2.3.1), 333µM each of dATP, dTTP, dGTP and dCTP (Promega), 1 unit of Taq polymerase (Cetus Amplitaq). DNA or cDNA samples were added and the PCR mix overlaid with paraffin oil. In general the cycle conditions were, one 5 minute 94°C incubation, followed by 25 to 40 cycles at 94°C for 45 seconds, 60°C for 60 seconds and 70°C for 90 seconds.

2.8.7 RNA Extraction

This method is a modification of that described by Chomczynski and Sacchi (1987). Tissue (50mg) was homogenised on ice with 1ml of guanidinium solution (2.4.5.6) in a 15ml glass/teflon homogeniser. Exactly 700µl of sample was then transferred to a fresh microfuge tube and 70µl 2M sodium acetate pH 4.0, 700µl phenol (2.4.5.7) and 140µl chloroform added. The tubes were mixed by inversion after each addition. The final mixture was shaken vigorously for ten seconds and centrifuged at 13 000g for 20 minutes at 4°C.

The aqueous phase was transferred to a new tube and 700µl of isopropanol added. The tubes were mixed and placed at -20°C for at least one hour. After centrifugation for 20 minutes at 13 000g, RNA pellets were resuspended in 300µl of guanidinium solution. The RNA was precipitated with an equal volume of isopropanol as above, washed three times with 70% ethanol and resuspend in 50µl DEPC treated hpH_2O (2.4.5.2). For short term storage RNA was stored at -70°C, for longer storage RNA was stored as a pellet "under ethanol" at -70°C prior to a final resuspension in 50µl DEPC treated hpH_2O. RNA was periodically assessed for degradation by electrophoresis (2.8.8).

2.8.8 RNA electrophoresis

RNA samples were electrophoresed in a 1.2% formaldehyde agarose gel. Agarose was melted in 63ml of DEPC treated hpH_2O and allowed to cool to 65°C, 17ml of formaldehyde was added and mixed, followed by 20ml of 5 x formaldehyde gel-running buffer (2.4.5.5).

The formaldehyde agarose gel was allowed to set in a fume hood for ~30 minutes. The well forming comb was removed and the gel placed into an electrophoresis cell

that was dedicated to RNA electrophoresis. The agarose gel was electrophoresed at 5 V/cm for 5 minutes prior to the loading of RNA samples.

RNA samples were denatured prior to electrophoresis, 4.5µl (up to 30µg RNA) of RNA sample was added to; 2.0µl 5 x formaldehyde gel-running buffer, 3.5µl formaldehyde, 10.0µl deionised formamide and 1.0µl EtBr (2.4.5.3). Samples were incubated at 65°C for 15 minutes, then quenched on ice. Samples were centrifuged briefly to deposit fluid and 2µl of 5 x formaldehyde gel loading buffer (2.4.5.4) was added. RNA was electrophoresed for approximately 2 hours at 70 V in 1 x formaldehyde gel-running buffer.

2.9 SEMI-QUANTITATIVE PCR

2.9.1 cDNA reaction

Total RNA was extracted as described (2.8.7). Total RNA, 5µl (up to 1µg), was heated to 70°C for 5 minutes and quenched on ice. Denatured RNA was then added to 15µl of reaction mix to give final concentrations of 50mM KCl, 10mM Tris-HCl (pH 8.3), 4.5mM $MgCl_2$, 0.01% gelatine (2µl 10 x PCR buffer, 1µl of 50mM $MgCl_2$ plus 8.4µl hpH_2O), 0.2µM oligo dT primer (IDT), 10mM DTT, 1mM each of dATP, dCTP, dGTP, dTTP (Promega) with 1.8 units of AMV reverse transcriptase (Promega) and 8 units of RNAsin (Promega) per reaction. The reaction mix was incubated at 42°C for 1 to 2 hours. Samples were stored at -80°C until needed.

2.9.2 Semi-quantitative cytokine PCR conditions

Semi-quantitative PCR was carried out in a total volume of 30µl. The final concentration of the reaction components were 1 x PCR buffer (2.4.4.2), 2 to 2.5mM $MgCl_2$ (depending on primers used), 0.2µM of each sense and anti-sense primer

(2.3.1), 0.167μM each of dATP, dTTP and dGTP, 8.3μM dCTP (Promega), 1 unit of Taq polymerase (Cetus Amplitaq) and 0.05pM [α^{32}p] dCTP (3000Ci/mM). Samples were overlaid with paraffin oil. For the assessment of *in vivo* cytokine mRNA expression, all samples were measured in duplicate.

Cycle conditions were, one 5 minute 94°C incubation, followed by 25 cycles at 94°C for 45 seconds, 60°C for 60 seconds and 70°C for 90 seconds.

2.9.3 Semi-quantitative β-actin PCR conditions

Total mRNA concentration was estimated using primers for β-actin. Conditions of β-actin PCR differed from cytokine detection due to the increased concentration of β-actin mRNA compared to cytokine mRNA. The concentration of dCTP was increased to 16.6μM. Radiolabelled [α^{32}p] dCTP was decreased to 0.014pM. Other reaction conditions were as described in section 2.9.2 with 2mM $MgCl_2$. Cycle conditions were the same as for cytokine semi-quantitative PCR.

2.9.4 Electrophoresis of PCR products

Following PCR, 10μl of DNA loading buffer (2.4.4.1) was added to each sample and mixed. PCR products were resolved by electrophoresis (2.8.1) of 15μl of PCR product/loading buffer mix on a 5% acrylamide gel (2.8.1). Electrophoresis conditions were at 150 V for 30 minutes in a Mini-Protean II (Bio-Rad) or 90 minutes in a Protean II (Bio-Rad) gel electrophoresis tank, electrophoresis buffer was 1 x TBE (2.4.4.3).

2.9.5 Quantitation of PCR product

Following electrophoresis, PCR gels were dried and autoradiographs taken (2.8.3). In initial studies, densitometry was performed on the autoradiographs for the determination of product yield. In later experiments, autoradiography was used to position acrylamide gels for excision of PCR product bands. Quantitation of the PCR product was performed by counting excised bands in a Hewlett Packard Cobra II gamma counter with energy window setting of 15-1000 keV. Counting of β–emitters under these conditions was approximately ~17% efficient. In latter experiments cpm obtained from each PCR product was determined by β-counting (Beckman LS 6500). Background radioactivity was corrected for by counting areas of equivalent size, to the band of interest, from the gel above or below the cytokine product band.

2.9.6 Normalisation to β-actin

β-actin mRNA levels in each total RNA sample was estimated by SQ-RT-PCR (2.9.3). The average β-actin value for all the samples assayed in a particular experiment was calculated. Each sample was then given a normalisation value by dividing the sample β-actin value by the average β-actin value. Individual cDNA samples were not used for SQ-RT-PCR if the sample β-actin value was ten fold lower or higher than the average β-actin value. The concentration of cytokine mRNA, as measured by SQ-RT-PCR, was then normalised to total mRNA concentration by dividing the cpm of the cytokine band (2.9.5) by that of the samples normalisation value. In later experiments β-actin values for all samples analysed were sufficiently similar so that compensation was not required.

CHAPTER THREE

DISEASE MODEL

Abstract

This chapter details the characterisation of a murine model of systemic candidiasis using a strain of *Candida albicans* (KEMH5) isolated from a patient with vulvo-vaginal candidiasis. In this model, CBA mice were consistently more sensitive to systemic candidiasis than BALB/c mice. The increased sensitivity of CBA mice was evident whether disease severity was assessed by mortality, histology or by quantitative yeast culture. Differences in the susceptibility of CBA and BALB/c mice became apparent within 24 hours of infection. This indicates that initial differences in sensitivities were not due to defects in the Ag specific response. This was supported by the finding that abscesses in both CBA and BALB/c mice was composed almost exclusively of non-specific neutrophils and macrophages, T-cells were generally not found within abscesses. Immunisation of CBA and BALB/c mice with a sublethal inoculum of viable *C. albicans* protected both strains of mice from subsequent re-challenge. However, immune CBA mice remained more sensitive to systemic candidiasis than naive BALB/c mice. This suggests that while recall Ag specific responses may be generated by immunisation, they are not sufficient to compensate entirely for the primary defect in CBA mice.

The predominate cell type found in the abscesses in the brain of BALB/c and CBA mice were F4/80$^+$ and or Mac-1$^+$ macrophages or microglia. MHC class II expression was high in brain abscesses in BALB/c mice. F4/80 expression was low in the kidneys of CBA mice. In contrast, Ia and F4/80 expression was high in abscesses in the kidneys of BALB/c mice. Yeast load was lower in the brain than in the kidneys. Additionally, yeast load was lower in the kidneys of BALB/c mice than CBA mice. Hence, there was a positive correlation between the presence of activated macrophages and increased yeast clearance. These data further reinforce the hypothesis that the principle mechanisms in host resistance to both primary and secondary systemic candidiasis are non-specific and that macrophages may have a major role in resistance to systemic candidiasis.

3.1 INTRODUCTION

Various animal models of candidiasis have been developed to study the spectrum of diseases caused by *Candida* sp. Models have been established for cutaneous candidiasis (Van Cutsem and Theinpont, 1971; Ray and Wuepper, 1976), genital candidiasis (Guentzel *et al.*, 1985), oropharyngeal and oesophageal candidiasis (Russel and Jones, 1973; Budtz, 1975; DeMaria *et al.*, 1976), gastrointestinal candidiasis (DeMaria *et al.*, 1976; Inoue *et al.*, 1988; Alexander *et al.*, 1990) and systemic candidiasis (Hector *et al.*, 1982; Inoue *et al.*, 1988; Alexander *et al.*, 1990; Ashman *et al.*, 1993). Models of systemic candidiasis have utilised the ability of *C. albicans* to translocate across the GIT (Inoue *et al.*, 1988; Alexander *et al.*, 1990). However, adult mice do not develop systemic candidiasis from intragastric inoculation unless immunosuppressed or treated with antibiotics (Russel and Jones, 1973; DeMaria *et al.*, 1976; Umenai *et al.*, 1979; Myerowitz, 1981). Consequently, most models of systemic candidiasis rely on an intravenous (*iv*) route of inoculation that mimics the process of catheter associated candidiasis (Hector *et al.*, 1982; Ashman and Papadimitriou, 1987; Ashman and Papadimitriou, 1988; Romani *et al.*, 1991a; Romani *et al.*, 1991b).

Hector and colleges (1982) described the sensitivity to systemic infection of six inbred strains of mice given an *iv* inoculation of *C. albicans*. Naive mice showed substantial differences in resistance to systemic candidiasis. C57Bl/6J and BALB/cByJ were the most resistant strains, A/J, C3H/HeJ and CBA/J strains demonstrated intermediate resistance and the DBA/2J mice were the most sensitive. Other studies have found that AKR and CBA/CaH mice were more sensitive than A/J, BALB/c, C57Bl/6, DBA/1, DBA/2 and SJL mice to *iv* inoculation of *C. albicans* when histological damage was assessed (Ashman *et al.*, 1993). CBA, A/J and DBA/2 mice were the most sensitive to infection when mortality was assessed (Ashman *et al.*, 1993). Differences in the resistance to systemic candidiasis have also been noted in inbred strains of mice

following immunisation (Hector *et al.*, 1982; Ashman and Papadimitriou, 1988). Therefore, strain related differences exist in the resistance of mice to primary and secondary systemic candidiasis.

The mechanisms that leads to the differential resistance patterns described above, have not been fully characterised. In many of the inbred strains of mice resistance patterns do not map to the H2 locus (Hector *et al.*, 1982). Hence, various mechanisms other than MHC mediated effects have been proposed, such as; complement defects (Thong and Ferrante, 1978; Heidenreich and Dierich, 1985; Linehan *et al.*, 1988; Ashman *et al.*, 1993), cell mediated defects (Ashman and Bolitho, 1993), non-cell mediated defects (Hurtrel and Lagrange, 1985), immune deviation (Romani *et al.*, 1993) and Ab responses (Moser and Domer, 1980). The contradictory nature of these results indicates the immune response to systemic candidiasis remains unclear. This study was initiated to address the role of T-cell and non T-cell derived cytokines in the resistance to systemic candidiasis. BALB/c mice were chosen as an example of a "healer" or resistant mouse and CBA mice were chosen as a "non-healer" or sensitive strain of mouse.

In order to compare immune responses such as cytokine mRNA patterns it was necessary to first characterise the model of systemic candidiasis used in this study as considerable variability in the pathogenicity of *C. albicans* strains has been demonstrated (Hector *et al.*, 1982; Ashman and Papadimitriou, 1987; Romani *et al.*, 1991b; Romani *et al.*, 1992b). Additionally, the application of immunohistochemistry in this study was designed to extend the findings of other studies by defining the cell populations that were found within abscesses caused by *C. albicans* infection.

3.2 SUMMARY OF MATERIALS AND METHODS

3.2.1 Materials

Mice of the strain BALB/c and CBA/CaH were obtained from the Animal Resources Centre, Murdoch University, Perth, Western Australia (2.1.1)

Candida albicans strain KEMH5 was obtained from Dr K Ott, Department of Pathology, King Edward Memorial Hospital for Women (2.1.2). KEMH5 was tested in the two inbred strains of mice used in this study, healer BALB/c and non-healer CBA/CaH mice.

3.2.2 Methods

Inoculation of mice was carried out as described in chapter 2 (2.5.2)

Quantitative yeast culture was performed as described in chapter 2 (2.5.3). Yeast colonisation levels are expressed as $\log_{10}$ cfu/g tissue. However, for the sake of simplicity, the full expression has been abbreviated to logs throughout the majority of this chapter.

Histology was performed as described in chapter 2 (2.6)

Immunohistology was performed as described in chapter 2 (2.7)

Statistical analysis was performed by use of a Mann-Whitney-U test.

PART 1

TISSUE DISTRIBUTION AND CLEARANCE RATES OF *C. ALBICANS* DURING SYSTEMIC CANDIDIASIS

It was necessary at the commencement of this study to characterise the tissue distribution and clearance patterns of mice following induction of systemic candidiasis with *C. albicans* strain KEMH5. This was necessary, both to determine virulence of this strain of *C. albicans* and to determine its preferential target organs. Secondary systemic candidiasis was studied to determine if CBA and BALB/c mice were protected from secondary infection by immunisation. Additionally, resistance to re-infection and *Candida* tissue distribution patterns were characterised to allow for the correlation of resistance to cytokine gene expression.

3.3 RESULTS PART 1

3.3.1 Quantitative yeast culture in acute primary systemic candidiasis

Mice were inoculated with either, $1x10^4$, $1x10^5$ or $1x10^6$ viable *C. albicans* blastospores. Uninfected control mice were inoculated with sterile non-pyrogenic saline. Four infected mice were sacrificed by cervical dislocation on each of days one-five for quantitative yeast culture. Three infected mice were sacrificed on day seven for quantitative yeast culture. Two uninfected mice were sacrificed on each day to ensure mice did not harbour *C. albicans* prior to the experiment. Control mice had no detectable levels of *C. albicans* (<2.6 $\log_{10}$ cfu/g tissue) in any of the tissues tested (data not shown).

3.3.1.1 Brain

BALB/c and CBA mice were inoculated with $1x10^4$ blastospores of *C. albicans* and tissue colonisation levels assessed (Figure 3.1a). Levels of *C. albicans* were low in the brain of both strains of mice, with less than 3 logs of *C. albicans* during the course of the first 7 days of infection. There was no significant difference in the level of colonisation in the brain between CBA and BALB/c mice over the seven day period. Yeast colonisation was below the limit of detection in the brain by day five in CBA mice and by day seven in BALB/c mice.

Non-healer CBA mice, inoculated with $1x10^5$ *C. albicans*, had significantly greater recovery of yeast from the brain on days two, four and five of infection compared to comparably treated healer BALB/c (Figure 3.1b). Tissue colonisation in CBA mice ranged from 1 to 2 logs higher than BALB/c mice. CBA mice, inoculated with $1x10^5$ viable *C. albicans* blastospores, had a 10 to 1000 fold increase in colonisation levels in the brain compared to CBA mice given a $1x10^4$ inoculum. In contrast, colonisation levels in the brain of BALB/c mice were similar for both inoculum sizes (Figure 3.1a and 3.1b).

When inoculated with $1x10^6$ *C. albicans*, in contrast to the previous inoculations ($1x10^4$ and $1x10^5$), CBA mice did not significantly decrease the level of yeast colonisation in the brain over the course of the infection (Figure 3.1a-c). The level of yeast colonisation in the brain of BALB/c mice was reduced approximately 35 fold from day five to seven (Figure 3.1c). Peak colonisation levels in the CBA mice were approximately 31 fold higher than those seen in the BALB/c mice. CBA mice had significantly higher yeast load in the brain than BALB/c mice at all time points with the exceptions of day four (Figure 3.1c).

3.3.1.2 Heart

There was only minimal levels of yeast colonisation in the heart of CBA and BALB/c mice following a $1x10^4$ inoculum of *C. albicans* (Figure 3.2a). Tissue colonisation levels did not rise above 2.1 logs in either strain of mouse, with many mice having no detectable *C. albicans* in homogenates of heart tissue. Additionally, there was no significant difference in the level of tissue colonisation in hearts of CBA compared to BALB/c mice. A higher inoculation of $1x10^5$ had no additional effect on tissue colonisation levels in the heart of BALB/c mice and only minimal effect on yeast numbers in the heart of CBA mice (Figure 3.2b). A significant difference in tissue colonisation between CBA and BALB/c mice was seen only on day one.

Increasing the inoculum size to $1x10^6$ viable *C. albicans* blastospores had no additional effect on the recovery of yeast from the heart of infected BALB/c mice (Figure 3.2c). There was similar levels of colonisation in the heart of BALB/c mice given either a $1x10^4$, $1x10^5$ or $1x10^6$ inoculation of *C. albicans*. In contrast, tissue colonisation in the heart of CBA mice was up to 200 fold higher in mice receiving a $1x10^6$ compared to a $1x10^5$ inoculum (Figure 3.2b and c). Additionally, the level of tissue colonisation in the heart of CBA mice was significantly higher than that detected in BALB/c mice for the first 3 days of infection.

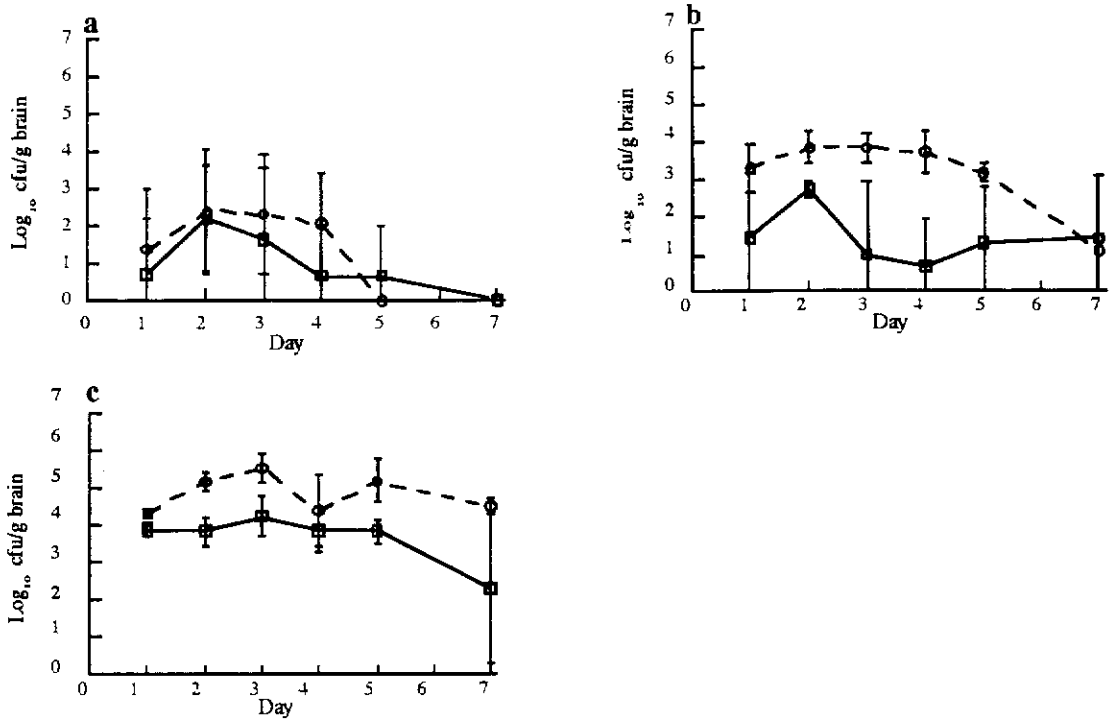

Figure 3.1 Colonisation levels of *C. albicans*, $\log_{10}$ cfu/g, in the brain of BALB/c (—□—) and CBA (- -○- -) mice as determined by quantitative yeast culture following a; a) $1x10^4$, b) $1x10^5$ or c) $1x10^6$ *iv* inoculum of *C. albicans*. Values are mean ± sd of n=4 mice from day one-five and n=3 on day seven.

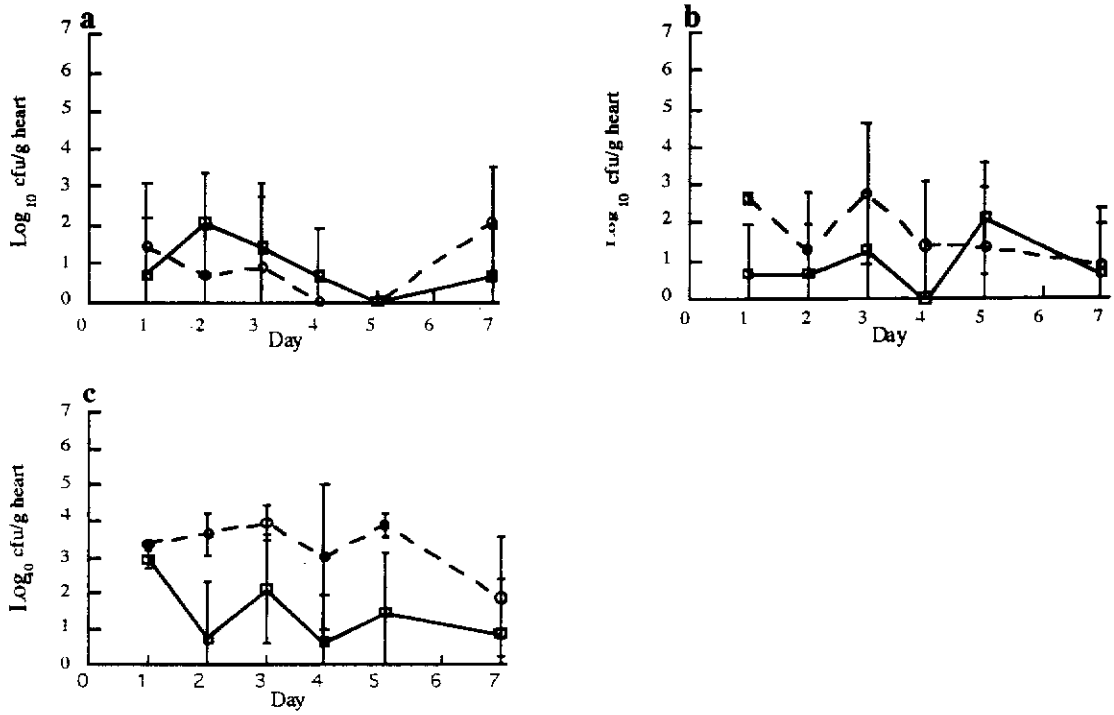

Figure 3.2 Colonisation levels of *C. albicans*, $\log_{10}$ cfu/g, in the heart of BALB/c (—□—) and CBA (- -○- -) mice as determined by quantitative yeast culture following a; a) $1x10^4$, b) $1x10^5$ or c) $1x10^6$ *iv* inoculum of *C. albicans*. Values are mean ± sd of n=4 mice from day one-five and n=3 on day seven.

3.3.1.3 Kidney

BALB/c and CBA mice were inoculated with $1x10^4$ viable *C. albicans* blastospores. CBA mice had significantly greater levels of colonisation of the kidney than BALB/c mice for the first 2 days of infection (Figure 3.2a). Peak cfu were recovered on day two in both CBA and BALB/c mice. However, peak colonisation in BALB/c mice was approximately 70 fold lower than CBA mice. Additionally, tissue colonisation was over 600 fold higher in CBA mice than BALB/c mice on day one. By day three, CBA mice had substantially cleared the yeast load such that both strains of mice had similar levels of colonisation from day three to day seven (Figure 3.1a).

With an inoculum of $1x10^5$ blastospores (Figure 3.1b), the increased level of colonisation in the kidney reflected the increased inoculum size in both strains of mice. Peak colonisation in the kidney was seen in the CBA mice at day two (4.93 logs). Maximum colonisation was seen at day one in the BALB/c mice (3.92 logs). The CBA mice had significantly greater colonisation of the kidney than BALB/c mice on days two and seven. Colonisation fluctuated in the BALB/c mice which had undetectable levels of yeast by day seven. In contrast, CBA mice had increasing yeast colonisation from day one to two, with a trend to reduced tissue colonisation to day five. However, in CBA mice, there was a sharp increase in yeast colonisation from day five to seven, with colonisation levels increasing over 360 fold. A similar reduction in yeast load, followed by a rapid increase in tissue colonisation of the kidney, was seen in an earlier experiment with a $3x10^5$ inoculum of *C. albicans*. In that experiment, tissue colonisation was 6.3 logs in the kidney at day four and had reduced 22 fold to 5 logs by day seven. Colonisation levels subsequently increased in the kidneys of treated CBA mice to 6.4 logs on day ten, a 27 fold increase in yeast load from day seven. This phenomenon was only evidenced in the kidney of CBA mice and was not seen in other tissues or in BALB/c mice.

CBA mice that received an inoculum of $1x10^6$ viable *C. albicans* blastospores, had peak colonisation, over 6 logs, in the kidneys at day three. In BALB/c mice, colonisation of the kidney was greatest, over 5 logs, on days one and two post inoculation. CBA mice had significantly higher yeast colonisation in the kidneys compared to BALB/c mice from days one-four and on day seven. Quantitative yeast culture was not performed on BALB/c mice on day five. The level of colonisation was substantially reduced in the kidneys of BALB/c mice from day four to seven. In contrast, tissue colonisation was not substantially reduced in the kidneys of CBA over the course of the first seven days.

3.3.1.4 Liver

CBA and BALB/c mice inoculated with $1x10^4$ viable *C. albicans* blastospores had minimal yeast colonisation in the liver as determined by quantitative yeast culture. Average yeast load was generally below 2 logs (Figure 3.4a) and colonisation levels were similar in both strains of mice. Similar results were evidenced when the inoculum size was increased to $1x10^5$ viable *C. albicans*. Both strains of mice had similar patterns of tissue colonisation with a significant difference between the two strains of mice evident only on day two and three. Levels of tissue colonisation in BALB/c and CBA mice, inoculated with $1x10^5$ viable *C. albicans*, were initially higher, by up to 350 fold, than from a $1x10^4$ inoculum. However, by day seven, yeast load resulting from the $1x10^5$ inoculum was similar to that developed from a $1x10^4$ inoculum.

Tissue colonisation was assessed in the livers of CBA and BALB/c mice following a $1x10^6$ inoculum of viable *C. albicans* blastospores. In CBA mice there was an increase of over 10 fold in the level of tissue colonisation compared to the $1x10^5$ inoculum for the first 3 days of infection (Figure 3.4b and c). CBA and BALB/c mice failed to clear the $1x10^6$ inoculum as effectively as those given a $1x10^5$ inoculum of

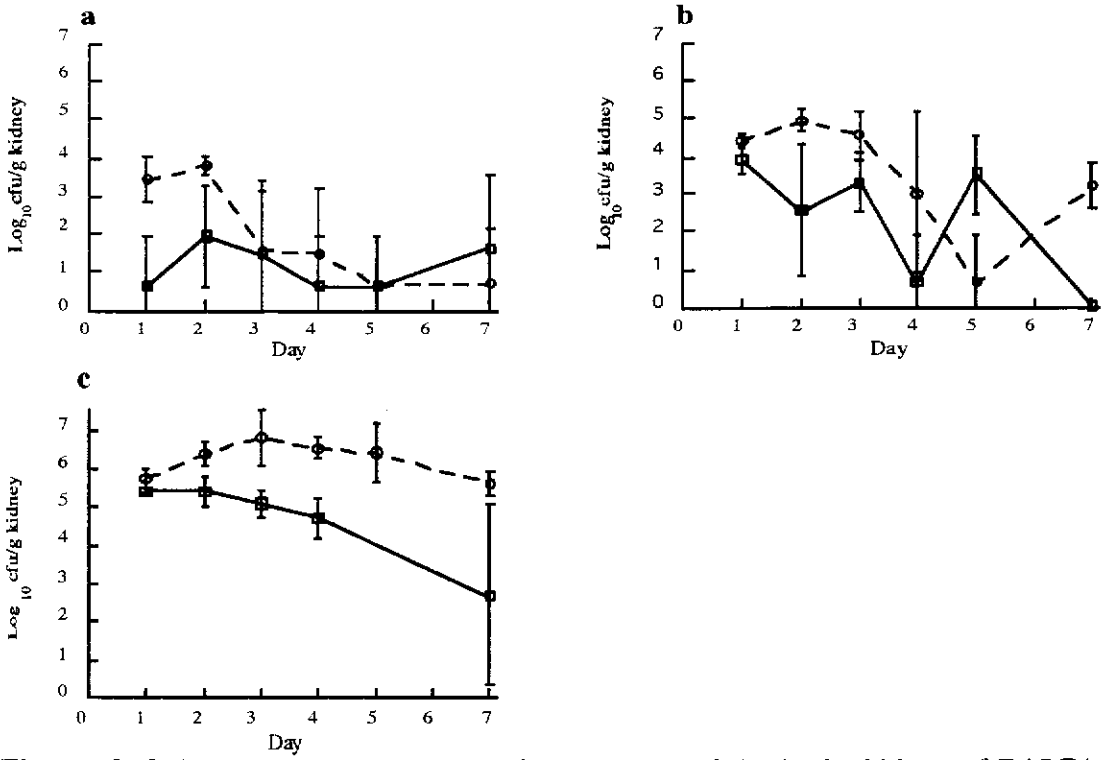

Figure 3.3 Colonisation levels of *C. albicans*, $\log_{10}$ cfu/g, in the kidney of BALB/c (—□—) and CBA (- -○- -) mice as determined by quantitative yeast culture following a; a) $1x10^4$, b) $1x10^5$ or c) $1x10^6$ *iv* inoculum of *C. albicans*. Values are mean ± sd of n=4 mice from day one-five and n=3 on day seven.

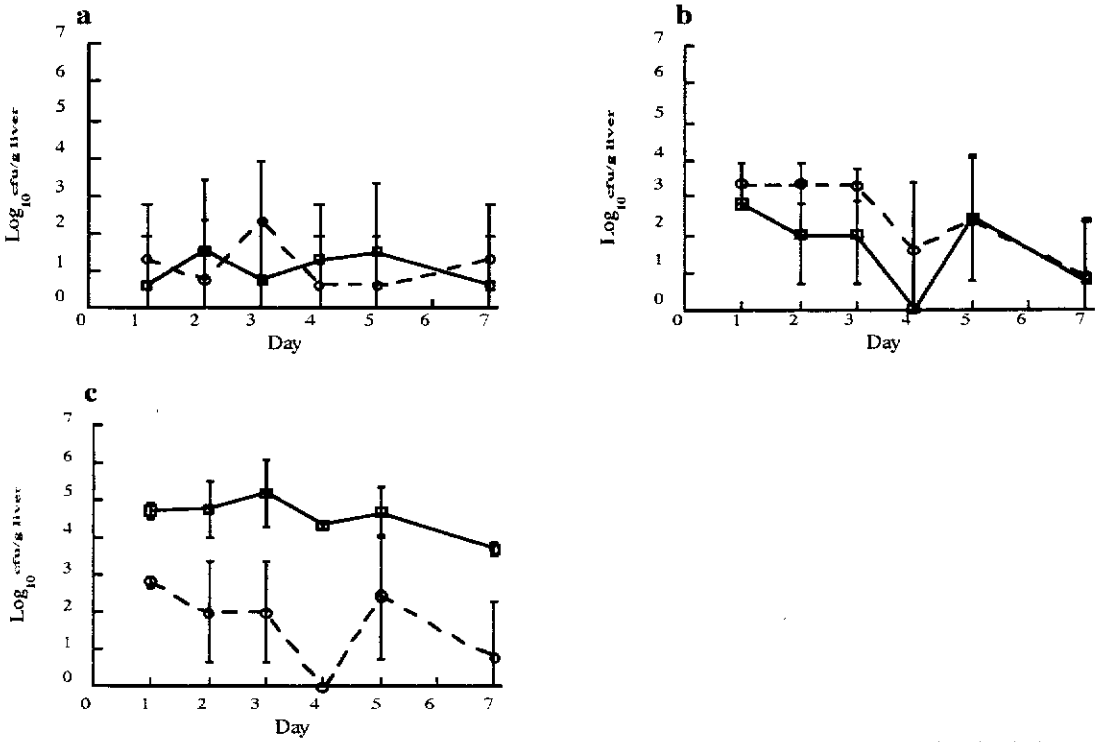

Figure 3.4 Colonisation levels of *C. albicans*, $\log_{10}$ cfu/g, in the liver of BALB/c (—□—) and CBA (- -○- -) mice as determined by quantitative yeast culture following a; a) $1x10^4$, b) $1x10^5$, or c) $1x10^6$ *iv* inoculum of *C. albicans*. Values are mean ± sd of n=4 mice from day one-five and n=3 on day seven.

viable *C. albicans* (Figure 3.4b and c). The level of tissue colonisation was significantly greater in the liver of CBA mice than BALB/c mice for the first 7 days of infection following a $1x10^6$ inoculum.

3.3.1.5 Lung

Tissue colonisation was assessed in the lungs of CBA and BALB/c mice following an inoculation of $1x10^4$ of viable *C. albicans* blastospores. Tissue colonisation was minimal in both strains of mice with an inoculum of this size (Figure 3.5a). A 10 fold increase in inoculum dose to $1x10^5$ viable *C. albicans* blastospores had only a marginal effect on increasing the number of recoverable yeast from the lungs of infected mice (Figure 3.5b). Tissue colonisation trends were the same for both strains of mice. There was no significant difference between the level of yeast colonisation in the lungs of BALB/c and CBA mice given a $1x10^5$ inoculum of *C. albicans*.

Increasing the inoculum size to $1x10^6$ substantially increased the yeast colonisation in the lungs of infected CBA mice. CBA mice had significantly higher levels of colonisation in the lungs during the first 7 days of infection than BALB/c mice. The BALB/c mice had an initial high yeast load on day one, but rapidly reduced yeast numbers below the level of detection by day seven. In contrast, CBA mice did not significantly alter the level of yeast load in the lungs over the course of the 7 days. Tissue colonisation levels remained above 3 logs in CBA mice at day seven.

3.3.1.6 Spleen

Mice inoculated with $1x10^4$ *C. albicans* blastospores had minimal yeast colonisation in the spleens. There was no significant difference in tissue colonisation between the two strains of mice during the course of the seven day period (Figure 3.6a). When the inoculum size was increased to $1x10^5$, peak tissue colonisation was increased by over

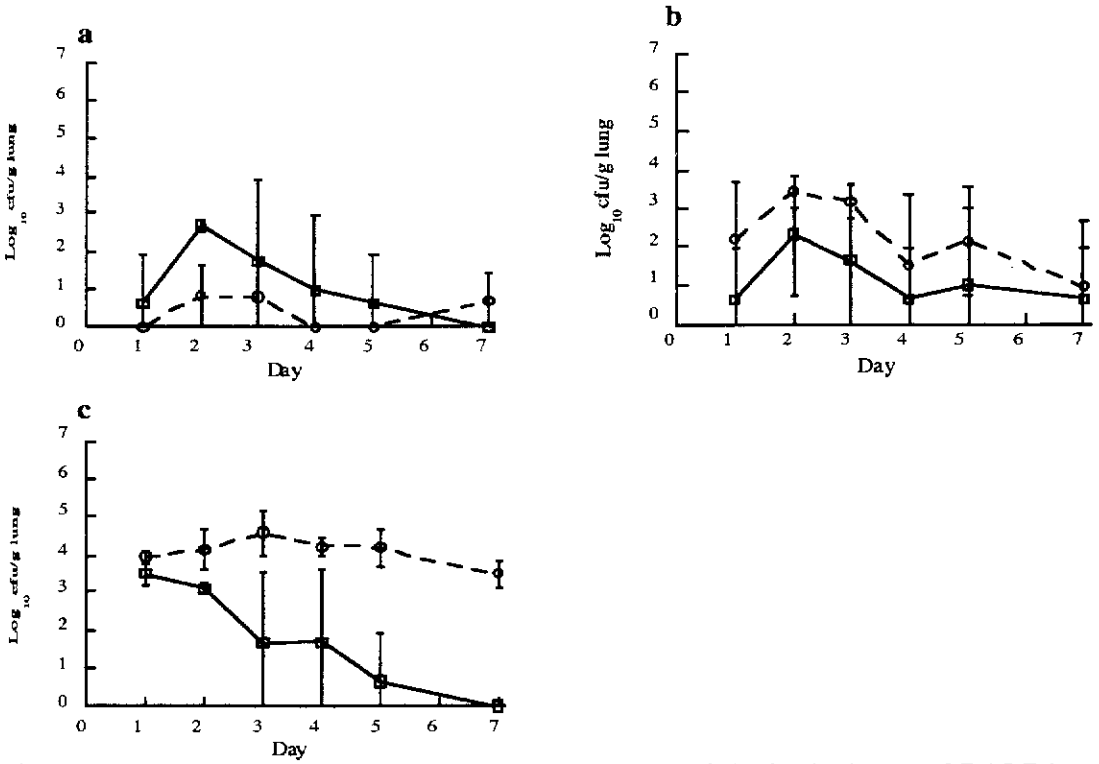

Figure 3.5 Colonisation levels of *C. albicans*, $\log_{10}$ cfu/g, in the lungs of BALB/c (—□—) and CBA (- -○- -) mice as determined by quantitative yeast culture following a; a) $1x10^4$, b) $1x10^5$ or c) $1x10^6$ iv inoculum of *C. albicans*. Values are mean ± sd of n=4 mice from day one-five and n=3 on day seven.

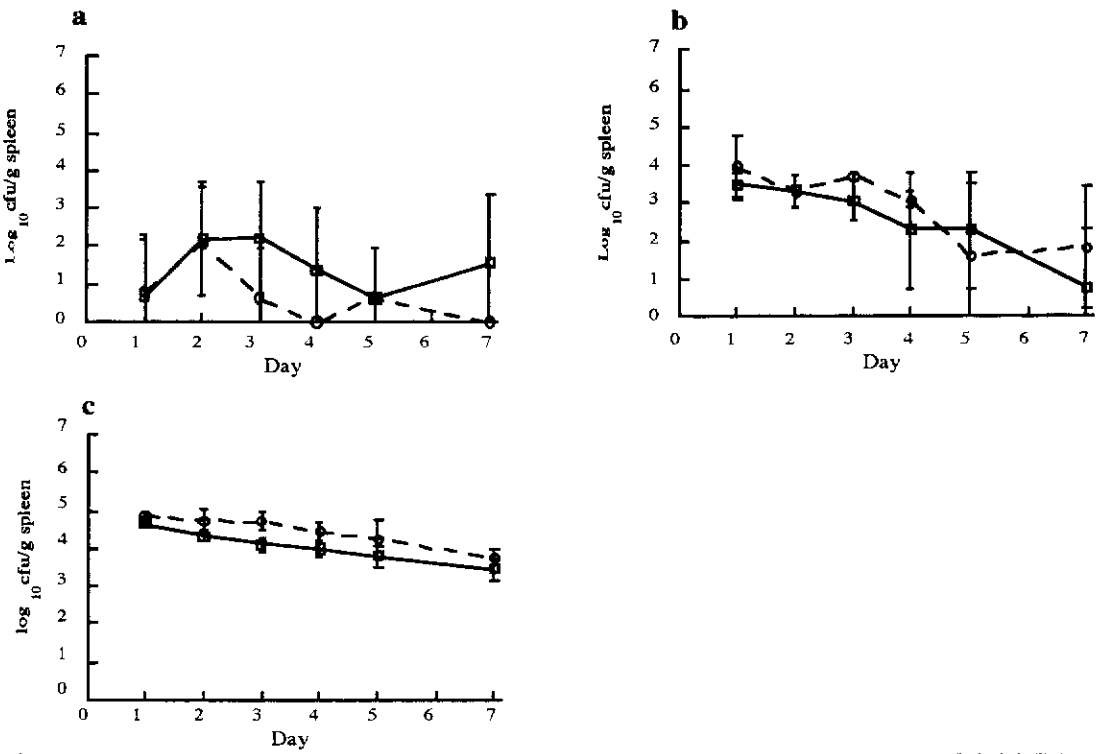

Figure 3.6 Colonisation levels of *C. albicans*, $\log_{10}$ cfu/g, in the spleen of BALB/c (—□—) and CBA (- -○- -) mice as determined by quantitative yeast culture following a; a) $1x10^4$, b) $1x10^5$ or c) $1x10^6$ *iv* inoculum of *C. albicans*. Values are mean ± sd of n=4 mice from day one-five and n=3 on day seven.

10 fold and in some cases over 1000 fold in CBA mice, and 500 fold in BALB/c mice (Figure 3.6a and b). The colonisation levels in the BALB/c and CBA mice closely paralleled each other in both amplitude and duration. However, CBA mice had significantly higher levels of yeast colonisation in the spleen than BALB/c mice on day one and three. In both strains of mice the yeast load was cleared by a factor of 100 to 500 fold over the course of seven days.

Increasing the inoculum size to $1x10^6$ substantially increased the yeast colonisation in the spleens of infected CBA and BALB/c mice (Figure 3.6b and 3.6c). However, in contrast to the $1x10^5$ inoculum, mice challenged with a $1x10^6$ inoculum had only moderate reduction in yeast colonisation in the spleen over the first 7 days of infection (Figure 3.6b and c). Additionally, CBA mice had a significantly higher yeast load in the spleen than BALB/c mice on days one to four. However, as was seen with the $1x10^4$ and $1x10^5$ inoculums, the rate of yeast clearance in the spleens of CBA and BALB/c mice inoculated with $1x10^6$ *C. albicans* were similar.

3.3.2 Quantitative yeast culture in secondary systemic candidiasis

Previous reports on the susceptibility of BALB/c and CBA mice to secondary systemic candidiasis have indicated that BALB/c mice have only a minimal increase in resistance to re-infection, whereas CBA mice have a substantial increase in their resistance to re-challenge (Hector *et al.*, 1982; Ashman and Papadimitriou, 1988). To ascertain if this was the case in our model, BALB/c and CBA mice were immunised and re-challenged 22-25 days later.

BALB/c mice were immunised by an *iv* injection of $1x10^5$ viable C. *albicans* blastospores, naive mice received sterile non-pyrogenic saline. Immunised and naive BALB/c mice were inoculated *iv*, 25 days later with $1x10^6$ viable *C. albicans* blastospores. Previous experiments with a $1x10^5$ immunisation of *C. albicans*

blastospores in CBA mice resulted in a mortality rate of 73% from day 21 to 26 (data not shown). Consequently, CBA mice were immunised by an *iv* injection of $1x10^4$ viable *C. albicans* blastospores, naive mice received sterile non-pyrogenic saline. Immunised and naive CBA mice were inoculated *iv*, 22 days later with $1x10^6$ viable *C. albicans* blastospores. Immunised CBA and BALB/c mice were sacrificed at day one and four, naive CBA and BALB/c mice were sacrificed at day four only. Tissue colonisation levels in infected mice was assessed by quantitative yeast culture.

Immunised BALB/c mice had a significantly lower level of *C. albicans* colonisation in the brain and spleen than naive BALB/c mice. However, there was a trend to lower yeast load in all the tissues of immunised compared to naive BALB/c mice (Table 3.1). Immunisation of CBA mice resulted in significant protection from re-challenge as assessed by mortality. Mortality of naive mice was 80% over the first four days, with 0% mortality seen in the immunised mice (Table 3.2). CBA mice that survived a $1x10^5$ immunising inoculum of *C. albicans* were also protected from re-challenged with $1x10^6$ viable *C. albicans* (data not shown). In contrast to immune BALB/c mice, there was a trend to increased yeast load in the brain and kidney of immune CBA mice from day one to day four, with stable yeast load in the other tissues (Table 3.2). While immunisation protected mice from secondary systemic candidiasis, immunised CBA mice remained more sensitive to systemic candidiasis than naive BALB/c mice (Table 3.2).

Tissue	Immune BALB/c day one	Immune BALB/c day four	Naive BALB/c day four
Brain	3.9 ± 0.2	3.9 ± 0.4	5.4 ± 0.6 ‡
Heart	3.4 ± 0.4	1.1 ± 1.5	3.5 ± 0.7
Kidney	5.9 ± 0.8	4.8 ± 0.7	6.1 ± 1.6
Liver	4.0 ± 0.6	2.8 ± 1.8	3.7 ± 0.7
Lung	3.8 ± 0.5	2.6 ± 2.1	4.1 ± 1.0
Spleen	3.4 ± 0.4	3.0 ± 0.4	4.3 ± 0.2 ‡
Mortality day four		0/6	0/6

Table 3.1 Yeast colonisation levels in tissues during primary and secondary infection with *C. albicans* in BALB/c mice. Immunisations were carried out as described in section 3.3.2. Values given are the mean and standard deviation of six mice per group.
‡ Significant difference between immunised BALB/c and naive BALB/c mice on day four.

Tissue	Immune CBA day one	Immune CBA day four	Naive CBA day four	Naive BALB/c day four
Brain	4.2 ± 0.3	6.05 ± 0.4	6.4*	5.2 ± 0.5 ‡
Heart	4.3 ± 0.8	3.9 ± 0.5	4.6*	3.1 ± 0.5
Kidney	6.8 ± 0.2	7.8 ± 0.1	7.9*	6.3 ± 1.3 ‡
Liver	4.9 ± 0.2	4.6 ± 0.3	5.0*	4.4 ± 0.5
Lung	4.3 ± 0.2	4.7 ± 0.3	5.3*	4.4 ± 0.7
Spleen	4.6 ± 0.05	4.8 ± 0.2	4.8*	4.3 ± 0.2 ‡
Mortality day four		0/4	4/5	0/5

Table 3.2 Yeast colonisation levels in tissues during primary and secondary infection with *C. albicans* in CBA and BALB/c mice. Immunisations were carried out as described in section 3.3.2. Values given are the mean and standard deviation of $\log_{10}$ cfu/g tissue for four mice per immune group and five mice per naive group. Significant mortality was seen in the naive CBA mice compared to immune CBA mice.

* No standard deviation given as only one mouse survived to day four.

‡ Significant difference between immune CBA and naive BALB/c mice on day four.

3.4 DISCUSSION PART 1

In this chapter the initial characterisation of a murine model of systemic candidiasis in BALB/c and CBA mice has been described. Whilst other models of systemic candidiasis in mice have been developed (Myerowitz, 1981; Hector *et al.*, 1982; Ashman and Papadimitriou, 1987; Ashman and Papadimitriou, 1988), differences in the virulence of *C. albicans* strains (Hector *et al.*, 1982; Ashman and Papadimitriou, 1987; Romani *et al.*, 1991b; Romani *et al.*, 1992b), necessitated the characterisation of a new model. Virulence of the *C. albicans* strain KEMH5, tissue colonisation patterns of KEMH5, relative resistance of CBA and BALB/c mice to this strain of *C. albicans* and response of CBA and BALB/c mice to secondary infection with this strain of *C. albicans* were characterised.

During primary systemic candidiasis the acute infection was targeted as differences in susceptibility to systemic candidiasis become apparent early (Ashman and Papadimitriou, 1987). Yeast colonisation was consistently higher in the kidneys than in other organs as determined by quantitative yeast culture. The brain was the next most heavily colonised organ followed by the liver and spleen, then lungs and finally the heart. This pattern of tissue colonisation follows the trend seen in most animal models, in that, the kidney is typically the most affected organ in *iv* induced systemic candidiasis (Hector *et al.*, 1982; Levy *et al.*, 1985).

The differential targeting of *C. albicans* to organs of the host suggests that factors such as differential adherence (Calderone *et al.*, 1985; Brawner *et al.*, 1991), blood flow volume or resident host defence mechanisms regulate the level of colonisation in different tissues. Ashman and Papadimitriou (1987) found that *C. albicans* preferentially targets the liver and the lungs within 2 hours of *iv* injection of viable *C. albicans*. However, later in the infection (by 24 hours), the greatest level of tissue pathology was demonstrated in the heart, kidney and the brain. This suggests the liver

and lungs removes yeast form the blood, possibly by preferential adherence as well as volume of blood flow, but do not become infected with the yeast. Hence, organs such as the kidneys, which have lower traffic of yeast, appear to have a poorer innate defence mechanisms which allow for overgrowth of yeast. Therefore, high yeast load in the kidneys and the brain appears to be due to yeast replication at these sites, probably because these organs do not have a large resident phagocytic cell population like the spleen, liver and lungs.

CBA mice were consistently more sensitive to systemic candidiasis than BALB/c mice. These data confirm previous reports (Hector *et al.*, 1982; Ashman and Papadimitriou, 1987; Ashman *et al.*, 1993). The increased sensitivity of CBA mice was not due to increased traffic of *C. albicans* to the organs in CBA mice (Ashman and Papadimitriou, 1987). This suggests that differences in yeast colonisation in the tissues of CBA mice compared to BALB/c mice is due to different rates of *C. albicans* proliferation in affected tissue. The early nature of the difference in susceptibility implies that BALB/c mice have a more effective or more rapid innate response than CBA mice to systemic candidiasis. The kidney consistently had the highest yeast load. It is reasonable, therefore, to speculate that the kidney is responsible, at least in part, for dissemination of yeast to other organs and that control in this organ would increase resistance to systemic disease. It is possible, therefore, that BALB/c mice have a more rapid or effective innate response in the kidney than CBA mice.

From these studies it was apparent that CBA mice had three responses to infection that were demonstrated by varying the inoculum size. CBA mice given a $1x10^4$ inoculum cleared the infection within seven days, similar to that demonstrated by BALB/c mice. Additionally, a $1x10^4$ inoculum resulted in increased resistance to re-infection in CBA mice. When a "medium" inoculation of viable *C. albicans* was given *iv* ($1x10^5$ to

$1x10^6$ depending on the experiment), CBA mice reduced the yeast load in the acute infection. However, CBA mice treated in this fashion succumbed to chronic infection with increased yeast load and increased mortality. Finally, CBA mice given $1x10^6$ viable *C. albicans* succumbed, depending on the experiment, to a rapidly fatal acute infection, with death usually occurring within four days.

The above findings indicate that CBA mice are capable of controlling a suitably small inoculum with innate responses and that priming of the immune response may generate Ag specific responses that are protective in secondary infections. With a higher inoculation of *C. albicans* the innate immune responses were able to control infection in the acute phase of infection. However, the innate mechanism appeared to be overwhelmed in the chronic infection and mice succumbed to infection. Alternatively, increased mortality in chronic infection was due to the generation of an inappropriate Ag specific responses that was detrimental to the host.

The increased resistance of CBA and BALB/c mice to secondary infection demonstrates that immunisation is capable of increasing the resistance of both strains of mice to infection. Similar results have been demonstrated by others (Hector *et al.*, 1982; Ashman and Papadimitriou, 1988). However, it is not clear that these effects are Ag specific as Ag non-specific effects can also increase the resistance of mice to systemic candidiasis (Bistoni *et al.*, 1988; Vecchiarelli *et al.*, 1988). The increased resistance to infection, demonstrated in CBA mice, was not sufficient to induce a level of protection that equalled that seen in naive BALB/c mice. Therefore, the Ag specific or non-specific effects of immunisation were not sufficient to compensate for the primary defect in the resistance of CBA mice.

Therefore, in conclusion, the CBA mice appear to have a defect in the effectiveness of the initial innate response to primary systemic candidiasis. A sufficiently low inoculum of *C. albicans* can be cleared by CBA mice and lead to increased resistance

to secondary systemic candidiasis. However, an "intermediate" inoculum of *C. albicans* can lead to a fatal chronic infection after five to seven days that suggests a breakdown in the innate responses or an inappropriate Ag specific response. It is possible this period of increased mortality is related to the induction of a Th2 response (Romani *et al.*, 1993) possibly as a result of high Ag load at the induction of the T-cell response (Parish, 1972). A "high" *C. albicans* inoculum leads to rapid death in CBA mice probably due to complete breakdown in host resistance, with overload of yeast in critical organs, such as the kidneys and the brain. Finally, immunisation does protect CBA mice from secondary infection. However, immunisation was not sufficient to overcome the primary defect in the non-healer CBA mice.

PART 2

HISTOLOGY AND IMMUNOHISTOLOGY

3.5 RESULTS PART 2

Histological studies were undertaken in this chapter to determine the histopathological effects of *C. albicans* in target organs during systemic candidiasis. Tissues were stained by standard H&E and PAS techniques (2.5.4.1 and 2.5.4.2) to elucidate inflammatory cell infiltration patterns and *C. albicans* respectively. Additionally, immunohistochemistry was used to determine the surface phenotype of the cells infiltrating abscesses in the brain and the kidney.

3.5.1.1 Histology of primary systemic candidiasis

Histology was performed in at least three independent experiments with inoculation sizes of *C. albicans* from $1x10^5$ to $1x10^6$. Tissues were generally removed on day four for histology and stained with H&E and PAS techniques. Repeated experiments failed to find evidence for yeast infiltration or tissue damage in the spleen, liver, heart or lungs of infected BALB/c or CBA mice. This was evidenced even in mice with extensive yeast load (Figure 3.7 and 3.8). Tissue colonisation in the kidney seen in Figure 3.8 was 7.9 logs, tissue colonisation in the brain seen in Figure 3.7 was 6.4 logs. However, yeast was not seen in the spleen, liver and lungs with tissue colonisation levels of 4.8, 5.0 and 5.3 logs respectively.

The lack of histological evidence of infection was not a result of lower yeast load in the liver, lungs and spleen as inflammatory responses were readily detectable by histology in the brain and kidneys of mice with colonisation levels less than 5 logs (data not shown).

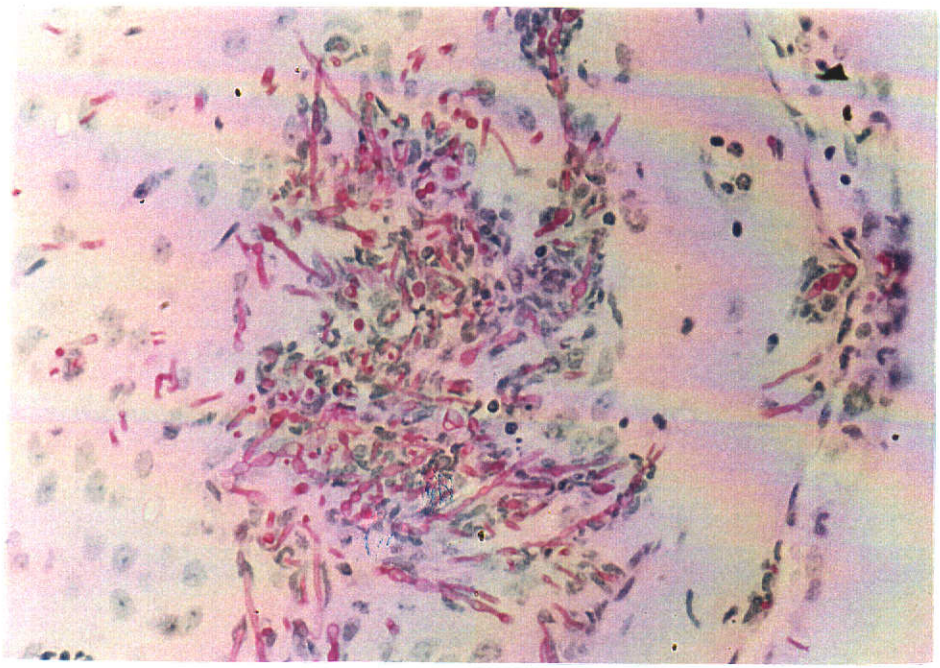

Figure 3.7 Photomicrograph (x200): PAS stain of infected brain from a CBA mouse taken four days after an *iv* inoculation of $1x10^6$ viable *C. albicans*. PAS technique stains *C. albicans* pink. *C. albicans* can be seen as the mycelial and yeast growth forms.

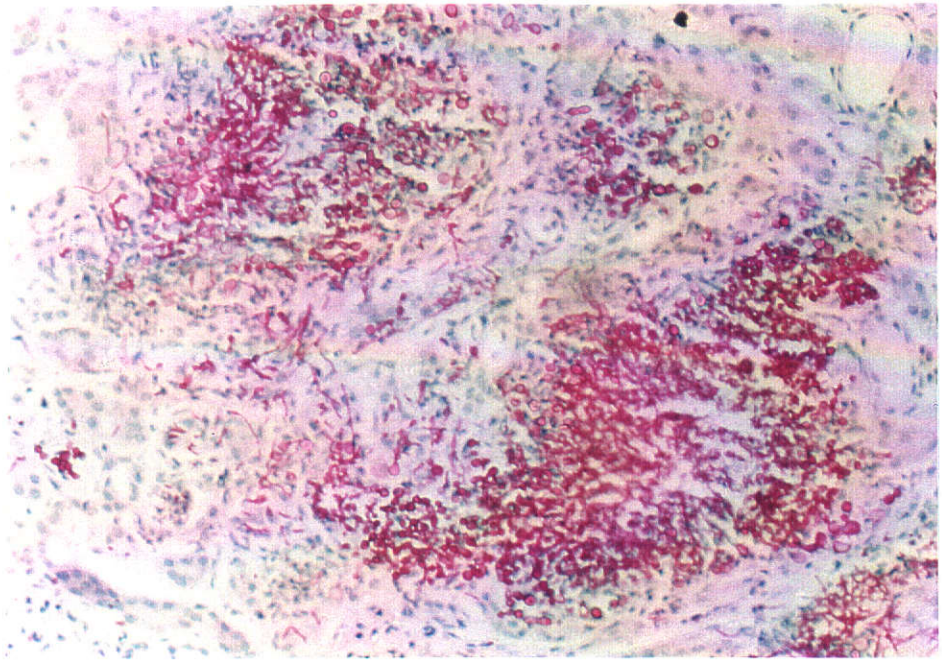

Figure 3.8 Photomicrograph (x100): PAS stain of infected kidney from a CBA mouse taken four days after an *iv* inoculation of $1x10^6$ viable *C. albicans*. PAS technique stains *C. albicans* pink. *C. albicans* can be seen as the mycelial and yeast growth forms. A large polymorphonuclear infiltrate is demonstrated in the abscess in the centre of the photomicrograph.

Yeast infiltration and tissue pathology was consistently greater in the kidneys than in the brain. This was evidenced in BALB/c and CBA mice (Figure 3.7, 3.8 and Table A.1 and A.2). In CBA mice the predominate immune infiltrate in the kidneys was polymorphonuclear, with the majority of the cells located in the foci of the infection, along with yeast and mycelial forms of *C. albicans* (Figure 3.8). Occasional cells could be seen with intracellular yeast, indicating active phagocytosis. Areas of necrosis were evident in the kidneys of CBA mice and less so in the kidneys of BALB/c mice. Similar inflammatory infiltrates were demonstrated in the kidneys of BALB/c mice to that of CBA mice. However, the kidneys of BALB/c mice had fewer (Table A.1 and A.2) and smaller abscesses than CBA mice.

Abscess formation in the brain of infected CBA mice was less frequent than in the kidneys. Abscesses in the brain were smaller than those seen in the kidney and had less inflammatory cells and less fungal elements (Figure 3.7, 3.8 and Table A.1). Similar abscess formation was seen in BALB/c mice, however the number of abscesses in BALB/c mice was consistently lower than those of CBA mice (Table A.1 and A.2). Mononuclear cells formed the major component of the inflammatory infiltrate in the brain of both CBA and BALB/c mice, although polymorphonuclear cells were present. Generally yeast in the brain tissue was confined to areas of inflammation and abscess formation, whereas in the kidney yeast were often found throughout the tissue. However, yeast was detected throughout the brain parenchyma in CBA mice given a high inoculum of $1x10^6$ (Figure 3.8).

3.5.1.2 Histology of secondary systemic candidiasis

CBA mice were immunised with an *iv* inoculum of $1x10^4$ viable *C. albicans* blastospores. Secondary systemic candidiasis was induced by re-challenge 22 days later with an inoculum of $1x10^6$ viable *C. albicans* blastospores *iv*. BALB/c were immunised with an *iv* inoculation of $1x10^5$ viable *C. albicans* blastospores. Secondary infection was established by re-challenge 25 days later with an inoculum of $1x10^6$ viable *C. albicans* blastospores *iv*. Tissues were removed from CBA and BALB/c mice four days after inoculation with $1x10^6$ viable *C. albicans* blastospores.

Fungal elements or evidence of immune infiltration was not detected in tissues other than the kidney and brain. In CBA mice there were fewer abscess, which were also smaller in size, than those found in naive mice (Table A.1). Additionally, fewer fungal elements were evident in the brain and kidneys of immunised CBA mice compared to naive CBA mice (Table A.1). Inflammatory infiltrates were minimal in the brain of immunised BALB/c mice and fungal elements were difficult to detect (Table A.2). Yeast cells were not detected in the kidneys of BALB/c mice at day four of secondary systemic candidiasis (Table A.2). Hence, immunised CBA mice had considerably higher yeast load and more prominent tissue destruction than immunised BALB/c mice. In addition, the level of yeast colonisation and tissue destruction was higher in immunised CBA mice than naive BALB/c mice. There was no obvious difference in the inflammatory infiltrates between immunised and non-immunised mice.

3.5.3 Immunohistochemistry

CBA mice were inoculated with $1x10^4$ viable *C. albicans* (KEMH5) in 200µl of sterile non pyrogenic saline, and given a further $3x10^5$ *C. albicans* in 200µl of sterile non pyrogenic saline 14 days later. At the time of secondary challenge naive CBA mice

were challenged with $3x10^5$ viable *C. albicans* blastospores. BALB/c mice were given similar treatment with the exception that $1x10^5$ *C. albicans* was used for immunisation. A total of twelve mice were used with a single uninfected mouse for each strain, three naive infected mice for each strain and two immune mice for each strain. All mice were sacrificed on day four. Multiple sections were taken at various "depths" (90μm apart) and the sections stained with a panel of mAb (Table 2.1). Typical staining patterns in spleens and lymph nodes were used as a control for each Ab (data not shown).

The interpretation of the immunohistology of the kidneys of both strains of mice was compounded by the presence of considerable endogenous peroxidase activity. Endogenous peroxidase activity was not present in the renal abseccess. Hence, much of the discussion of the inflammatory infiltrate has been confined the abscesses. However, this precludes in many cases, and particularily in CBA mice, a discussion of cells within the parenchyma of the tissue. In addition, as abscesses are characterised by a central area of necrosis in which many cells are dead it cannot be concluded, with certainty, that absence of specific staining is due to absence of a specific cell type or due to degradation of the Ag on the cell surface. The CD11b Ag, at least, appears to be refractory to this degradation as this was highly expressed in the kidneys of both strains of mice. Hence, the data from these studies is included in an appendix (A2-5) and it is required that interpretation of this data is seen as the interpretation of preliminary experiments only.

Abscess formation was considerably higher in the kidneys than the brain of infected CBA mice, this was evidenced in both the primary and secondary infection. Similar results were obtained in the BALB/c mice, with abscess formation being consistently higher in the kidney rather than the brain of mice with either primary or secondary systemic candidiasis. Although CBA and BALB/c mice had reduced abscess

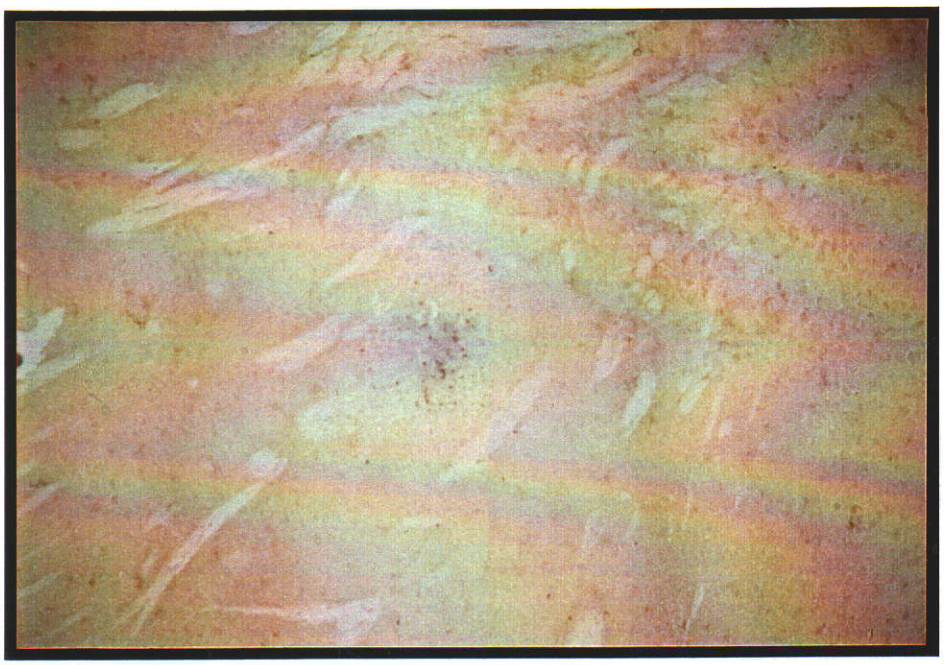

Figure 3.9 Photomicrograph (x100): Control staining using sheep anti-rat secondary Ab only. Photomicrograph shows an abscess in the brain of a CBA mouse at day four of primary systemic candidiasis. Non-specific staining, probably due to Ab binding to Fc receptors on immigrant phagocytic cells, can be seen in abscess at the centre of the photomicrograph.

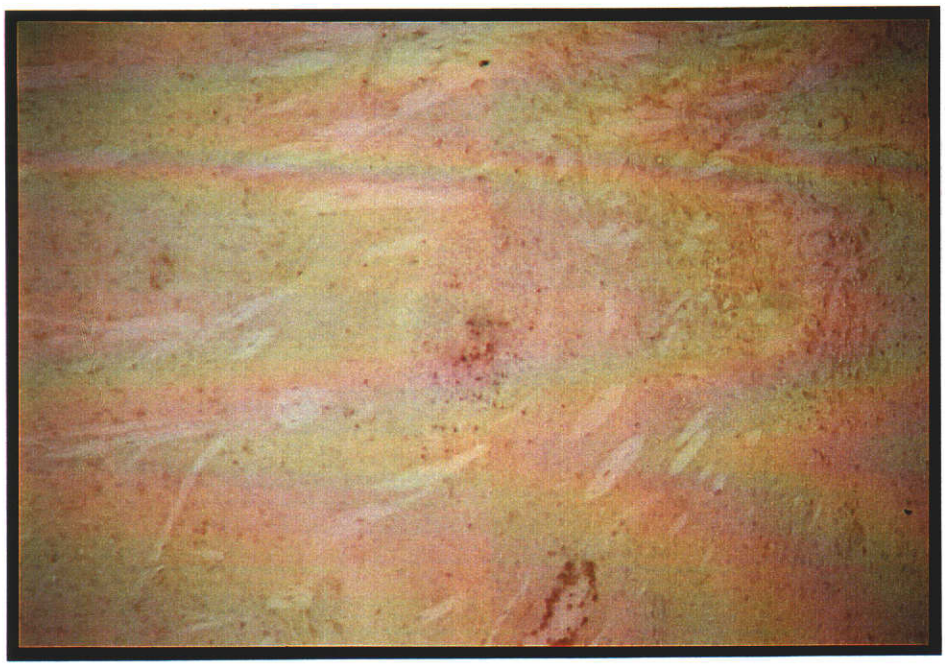

Figure 3.10 Photomicrograph (x100): CBA brain tissue stained for CD3 expression. Photomicrograph shows an abscess in the brain of a CBA mouse on day four of primary systemic candidiasis. CD3 staining in the abscess is similar to the non-specific Fc staining seen in figure 3.9. Specific staining for CD3 can be seen in the parenchyma surrounding the abscess and in blood vessels.

formation when immunised, immune CBA mice had significantly higher abscess formation than either naive or immune BALB/c mice.

3.5.3.1 CD3, CD5, CD4 and CD8 staining

Cells positive for the T-cell marker CD3 (Table 2.1) were detected in the brain of CBA mice during primary systemic candidiasis (Table A.1). However, $CD3^+$ cells were generally not found within the abscess of the brain (Figure 3.9). $CD3^+$ cells were most commonly found within blood vessels and scattered "lightly" through the parenchyma of the brain. $CD4^+$ cells were found in comparable numbers to $CD8^+$ cells. $CD3^+$ infiltration in the brain of naive CBA mice was similar to that seen in the brain of immunised CBA mice. $CD4^+$ cell numbers were similar to $CD8^+$ cells numbers in CBA mice with secondary systemic candidiasis (Table A.1). Finally, the distribution of $CD5^+$ cells in the brain of both naive and immune CBA mice was similar to that of CD3 expression (Table A.1).

In the kidneys of CBA mice, $CD3^+$ cells were seen predominantly in the parenchyma of the kidney and around the outside of the phagocytic infiltrate (Figure A.2). However, in occasional instances $CD3^+$ were seen within abscesses. $CD3^+$ cells were more prevalent in the kidney than in the brain in both CBA and BALB/c mice (Table A.1 and A.2). Both $CD4^+$ cells and $CD8^+$ cells were evident in the $CD3^+$ cells infiltrate with a possible bias to $CD8^+$ cells (Table A.1). Surprisingly, $CD5^+$ cells were more prevalent than $CD3^+$ cells (Figure A.3). This suggests that a subset of B-cells bearing the CD5 marker were present in the kidney of mice with systemic candidiasis. During secondary infection there was a modest infiltration of $CD3^+$ cells (Table A.1), which again was concentrated at the periphery of the phagocytic infiltrate. There was some evidence of an increased prevalence of $CD8^+$ cells compared to $CD4^+$ cells (Table A.1). There was no evidence of an increased infiltration of $CD3^+$ or $CD5^+$

cells in the kidneys of CBA mice with secondary systemic candidiasis compared with primary infection.

Detection of $CD3^+$ cells in the brain of infected BALB/c mice was considerably more difficult than in CBA mice. BALB/c mice had considerably fewer abscess in the brain than CBA mice and hence fewer infiltrating cells. However, $CD3^+$ cells could be detected in the brain of mice with primary systemic candidiasis (Table A.2). The pattern of infiltration was similar to that seen in the CBA mice with few $CD3^+$ present in the abscess. The majority of $CD3^+$ were scattered throughout the parenchyma and in the blood vessels. There was no evidence of an increase in $CD8^+$ cells compared to $CD4^+$ cells (Table A.2). During secondary infection there was very little abscess formation in the brain of BALB/c mice using this inoculation size and no $CD3^+$ cells were seen in the brain of these mice (Table A.2).

In the kidney of infected BALB/c mice there was modest infiltration of $CD3^+$ cells throughout the parenchyma of the tissue in both primary and secondary infection (Table A.2). The numbers of $CD3^+$ cells was similar to that seen in CBA mice (Table A.1). $CD3^+$ cells were generally not found within the abscesses in the kidneys of BALB/c mice, but in the parenchyma of the kidney and at the edges of phagocytic infiltrates. There was a slight predominance of $CD8^+$ cells compared to $CD4^+$ cells in the kidneys of BALB/c mice during primary and secondary infection systemic candidiasis (Table A.2). $CD3^+$ cells did not appear to be more prevalent in the kidneys of BALB/c mice during secondary infection compared to primary systemic candidiasis. However, abscess formation in the kidneys of BALB/c mice with secondary systemic candidiasis was very limited and most $CD3^+$ cells were found within the kidney parenchyma. As with the kidney of CBA mice, $CD5^+$ cells appeared to be more prevalent in the kidneys of BALB/c mice during primary and secondary systemic candidiasis than could be accounted for by CD3 staining.

3.5.3.2 F4/80 staining

F4/80 is a monoclonal Ab that is specific for a membrane associated antigen found almost exclusively on the surface of mature macrophages (Table 2.1). There was considerable inflammatory infiltration of F4/80$^+$ cells in the brain of CBA mice during primary systemic candidiasis (Figure 3.11 and Table A.1). The majority of F4/80$^+$ cells were confined to defined abscesses. However, F4/80$^+$ cells were also found throughout the parenchyma of infected brain. There was heterogeneity in the number of F4/80$^+$ cells in the brains of CBA mice during systemic candidiasis. In some cases, F4/80$^+$ cells were similar in number to Mac-1$^+$ cells, in others, Mac-1 staining was considerably higher than F4/80 staining. Numbers of F4/80$^+$ cells were consistently higher than CD3, CD4, CD5, or CD8 positive cells (Table A.1). Along with Mac-1$^+$ cells, F4/80$^+$ cells, comprised the bulk of cells detected within abscesses in the brain of CBA mice. Morphologically, F4/80$^+$ cells, were mostly small round cells typical of macrophages, however some cells had a more dendritic appearance, suggestive of microglia infiltration.

During secondary systemic candidiasis F4/80$^+$ and Mac-1$^+$ were again the predominate cell type found within abscesses in the brain of CBA mice (Table A.1). Cells had a similar morphology to those detected in the brain of mice during primary systemic candidiasis.

F4/80$^+$ cells were much less prevalent in the kidneys of CBA mice with primary systemic candidiasis than in the brain (Figure A.5, 3.11 and Table A.1). This was evident even though abscess formation was consistently higher in the kidney than in the brain. Only moderate infiltrates of F4/80$^+$ cells were detected in the kidneys of CBA mice during secondary systemic candidiasis (Table A.1). F4/80$^+$ cells were predominantly found at the periphery of abscesses and in the parenchyma, similar to the pattern seen for CD3$^+$ cells. However, due to heavy endogenous peroxidase

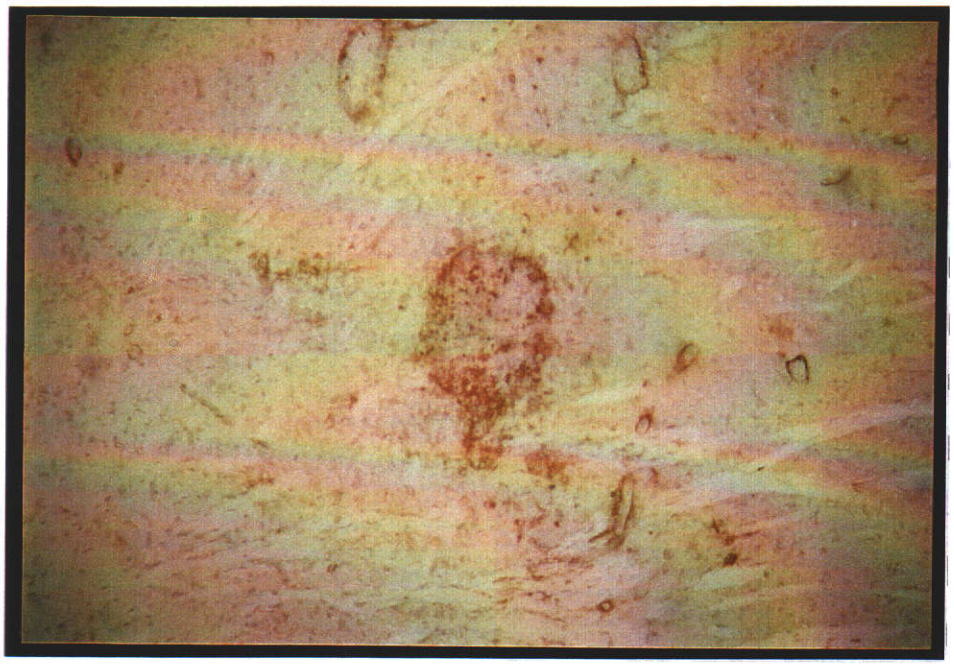

Figure 3.11 Photomicrograph (x100): CBA brain tissue stained for F4/80 expression. Photomicrograph shows an abscess in the brain of a CBA mouse on day four of primary systemic candidiasis. Cells expressing F4/80 can be seen predominantly at the periphery of the abscess and in the surrounding parenchyma.

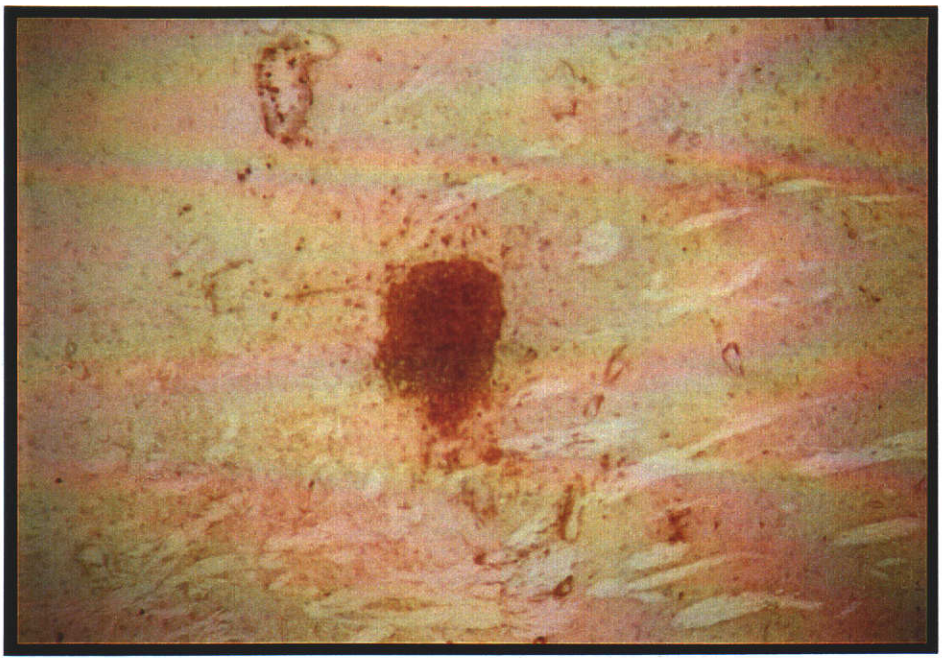

Figure 3.12 Photomicrograph (x100): CBA brain tissue stained for CD11b (Mac-1) expression. Photomicrograph shows an abscess in the brain of a CBA mouse on day four of primary systemic candidiasis. CD11b is expressed at high levels throughout the abscess. $CD11b^{+}$ cells can also be seen in the surrounding parenchyma and blood vessels

activity in the kidney it is not possible to state that F4/80+ cells were not found further into the parenchyma of the renal tissue. Morphologically, F4/80+ cells were similar to those found in the brain with most cells having the appearance of macrophages, however some cells had a more dendritic appearance. Similar results were obtained in the kidneys of CBA mice during secondary systemic candidiasis.

F4/80+ cells were readily detectable in the brain of BALB/c mice during primary systemic candidiasis (Figure 3.15 and Table A.2). The pattern of F4/80+ infiltration was similar to that seen in the CBA mice with the exception that abscess size was generally smaller in the BALB/c mice. In contrast to CBA mice, there was considerable infiltration of F4/80+ cells into the kidney of infected BALB/c mice (Table A.2). In the kidney of BALB/c mice the F4/80+ cells were not confined to the edges of the abscess and were equally as prevalent as Mac-1+ cells. However, abscess formation in the kidneys of BALB/c mice, at the inoculum size used in this experiment, was not as defined as that seen in the CBA mice. Hence, it is not possible to determine if these cells would reside at the periphery of the abscess if abscess size was larger and more neutrophils were present. Morphologically the F4/80+ cells were similar to those seen in CBA mice. Similar results were obtained in secondary infection in BALB/c mice (Table A.2). However, F4/80+ cells appeared to be more prevalent in secondary infection than was seen in primary infection in BALB/c mice.

3.5.3.3 Mac-1 staining

Mac-1 is a monoclonal Ab that is specific for the cell surface Ag CD11b which is present on the surface of macrophages, monocytes, granulocytes, B-cell subsets and NK cells (Table 2.1). Significant numbers of Mac-1+ cells were present in the brain of CBA mice with primary systemic candidiasis (Figure 3.12 and Table A.1). Mac-1+ cells were found in the abscess as well as throughout the parenchyma. Typically, Mac-1+ cell numbers, were similar to, or greater than F4/80+ cells. Morphologically,

Mac-1$^+$ cells, were all small round cells having the appearance of macrophages or neutrophils. There was no discernible difference in the number and pattern of Mac-1$^+$ cells infiltration in the brain of CBA mice with primary compared to secondary systemic candidiasis (Table A.1).

Mac-1$^+$ cells were the predominant inflammatory cell in the kidney of infected CBA mice and composed the bulk of the cells infiltrating abscesses (Figure A.6 and Table A.1). Mac-1$^+$ cells were equally prevalent in the abscess of CBA mice with primary systemic candidiais and secondary systemic candidiais (Table A.1). Morphologically, Mac-1$^+$ cells, were all small round cells having the appearance of macrophages and neutrophils. In BALB/c mice the number of Mac-1$^+$ cells found within the kidneys of infected mice was similar to the number of F4/80$^+$ cells (Table A.2). However, Mac-1$^+$ cells were lower in number than in CBA mice, which was a reflection of the reduced abscess formation in these animals. Mac-1$^+$ cells appeared to be more prevalent in the kidneys of BALB/c mice with secondary systemic candidiasis than during primary infection (Table A.2). However, abscess formation was difficult to detect in these mice and most cells were found within the parenchyma of the tissue. Mac-1$^+$ cell infiltration in the brain of BALB/c mice was similar in both primary (Figure 3.14 and Table A.2) and secondary systemic candidiasis to that seen in CBA mice (Figure 3.12 and Tables A.1 and A.2). Morphology of the cells in the brain and the kidney were similar. Most cells had the appearance of neutrophils or macrophages, however some F4/80$^+$ cells in the brain had a more dendritic appearance.

3.5.3.4 Ser-4 staining

Ser-4 is a monoclonal Ab that is specific for a subset of macrophages and binds the Ag sialoadhesin/SER (Table 2.1). There was no Ser-4 staining in the brain or the kidney

of either CBA or BALB/c mice. This was evidenced in both primary (Figure A.4) and secondary systemic candidiasis (Table A.1 and A.2).

3.5.3.5 MHC Class II staining

Staining for MHC class II (Ia) expression with the monoclonal Ab M5/114 was mostly confined to BALB/c mice which express the Ag determinant, I-A^d, at high levels (Table 2.1). Ia expression was detected in the brain and kidneys of CBA mice during primary and secondary systemic candidiasis. However, the Ia expression was low in CBA mice possibly as a result of the low level of I-E^k expression in these mice (Table 2.1 and 3.3). There was significant Ia expression in the brain of BALB/c mice with primary systemic candidiasis (Table A.2). Ia expression (Figure 3.13) was highest within defined abscesses in the region of Mac-1 and F4/80 expression (Figure 3.14-15). However, Ia was also up regulated in the surrounding tissue. Ia expression was considerably higher than could be explained by either Mac-1^+ and F4/80^+ (Figure 3.13, 3.14 and 3.15) alone and appeared to be higher than Mac-1 and F4/80 expression combined. There no discernible difference in the expression of MHC class II in the brain of BALB/c mice during secondary systemic candidiasis compared to primary systemic candidiasis.

Ia expression was high in the kidneys of BALB/c mice and as with the brain, was highest within abscesses but was also expressed in the parenchyma. This was in line with the findings of high numbers of F4/80^+ cells in the kidneys of BALB/c mice. Ia expression was higher in the kidneys of BALB/c mice during secondary infection compared to primary infection (Table A.2). In contrast, there was no apparent increase in Ia expression in the kidneys or brain of CBA mice during secondary systemic candidiasis compared to primary systemic candidiasis (Table A.1).

MHC class II expressing cells in the brain had various morphologies. Within abscesses the morphology of cells was similar to that obtained with Ab to F4/80 or Mac-1. In the parenchyma of the brain, morphology was less consistent. Cells varied from round to dendritic, with some cells having the appearance of neronal cells. In the kidney of infected animals there was considerable dendritic staining as well as the more regular staining seen with Mac-1.

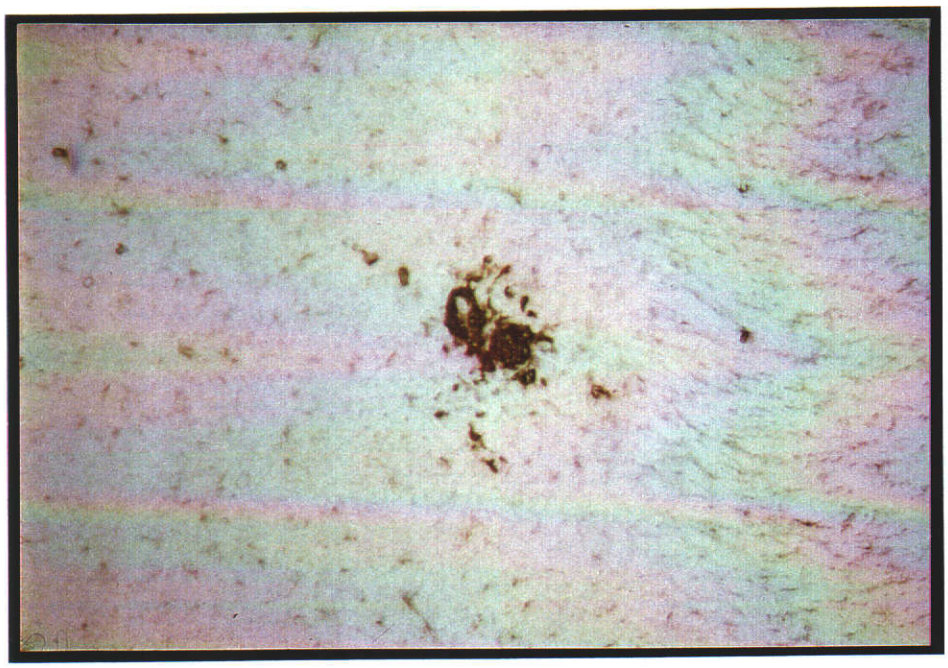

Figure 3.13 Photomicrograph (x100): BALB/c brain tissue stained for MHC class II (Ia) expression. Photomicrograph shows an abscess in the brain of a BALB/c mouse on day four of primary systemic candidiasis.

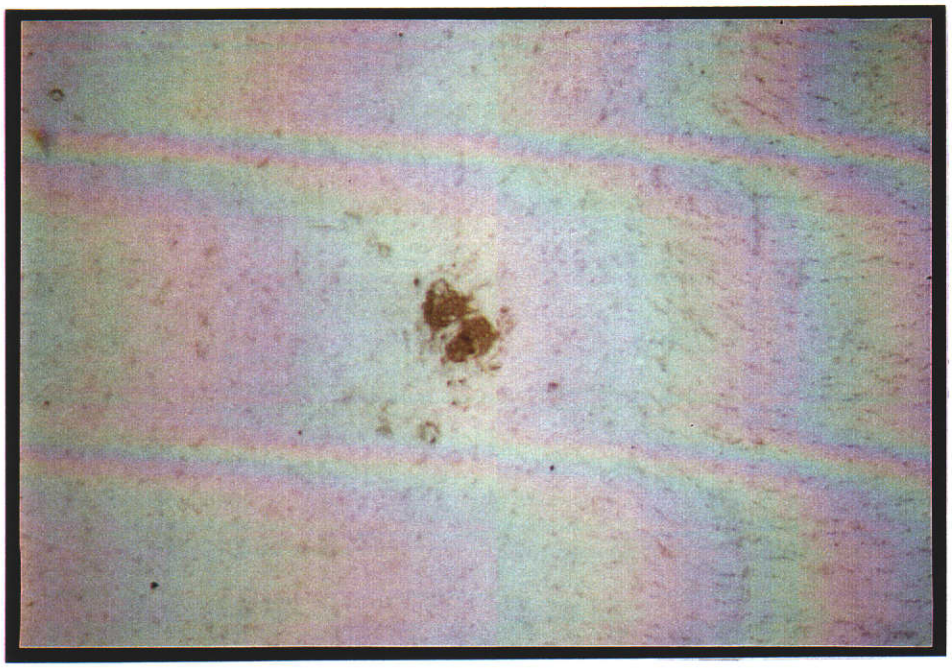

Figure 3.14 Photomicrograph (x100): BALB/c brain tissue stained for Mac-1 (CD11b) expression. Photomicrograph shows an abscess in the brain of a BALB/c mouse on day four of primary systemic candidiasis.

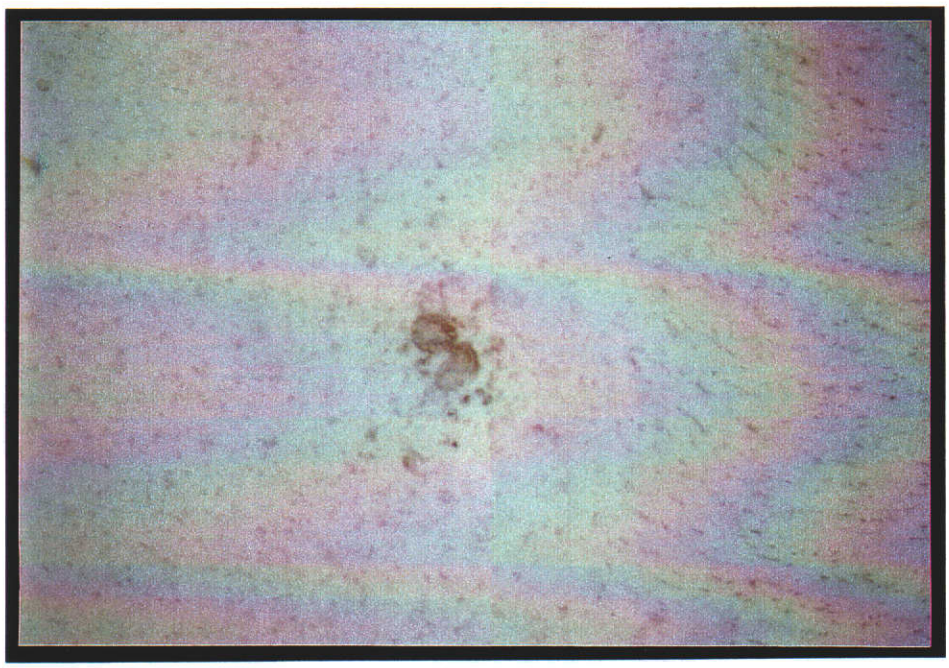

Figure 3.15 Photomicrograph (x100): BALB/c brain tissue stained for F4/80 expression. Photomicrograph shows an abscess in the brain of a BALB/c mouse on day four of primary systemic candidiasis.

3.6 DISCUSSION PART 2

Quantitative yeast culture and histology has demonstrated that *C. albicans* differentially affects target organs when administered *iv*. Analysis of tissue colonisation by quantitative yeast culture revealed significant yeast levels in the brain, kidney, spleen, liver, lungs and heart. However, histological analysis of affected organs shows *C. albicans* infection in the kidneys and brain only. The lack of histological evidence of infection in the spleen, liver, lungs and heart organs was not a result of reduced yeast load. Hence, quantitative organ culture identifies the presence of yeast in all organs in the absence of tissue pathology.

The lack of histopathological damage in the liver and lungs suggests rapid clearing of yeast from these organs by resident immune cells which prevents establishment of infection. This hypothesis is consistent with previous studies that have shown the liver and lungs are capable of removing large numbers of yeast from the circulation (Schwocho and Moon, 1981; Sawyer, 1990; Sawyer *et al.*, 1990). The continued presence of yeast in these organs, as determined by quantitative organ culture, would therefore appear to be a result of continued seeding from other organs. Levels of *C. albicans* in the heart are likely a reflection of candidemia or small focal infections as histological studies have failed to find *C. albicans* in this organ.

This study has found that inflammatory infiltrates found in the brain of infected mice vary from those that were found in the kidney of infected mice. Additionally, the inflammatory infiltrate found within the kidney of CBA mice was different to that found within the kidney of BALB/c mice. During primary infection in CBA mice the predominate immune infiltrate in the brain had markers for mature macrophages (Leenen *et al.*, 1994) and or microglia, both of which express the F4/80 Ag and CD11b (Leenen *et al.*, 1994). The $F4/80^+$ and $Mac\text{-}1^+$ cells in the brain were found both in overlapping and in non-overlapping areas suggesting the presence of at least

two cell populations, single F4/80$^+$ and Mac-1$^+$ cells as well as the possibility of double positive F4/80 and Mac-1 cells. Similar immune infiltrates were found in the brain of resistant BALB/c mice. The majority of cells within abscesses in the brain of BALB/c mice were MHC class II positive, suggesting these cells were activated and that the majority of Mac-1$^+$/F4/80$^-$ cells were not neutrophils. Hence, in both CBA and BALB/c mice there was significant infiltration of macrophages or microglia that, in BALB/c mice at least, expressed high levels of Ia. In the brain this was associated with rapid removal of yeast compared to the kidney and enhanced resistance to initial infection.

In the kidney of CBA mice the predominate cell type was Mac-1$^+$, F4/80$^-$ neutrophils. Abscesses had a peripheral zone of macrophages around a neutrophil aggregation suggesting a role for macrophages in the kidney. However, there was considerably less F4/80$^+$ cells than in the brain. There were occasional MHC class II positive and F4/80$^+$ cell with a dendritic appearance, consistent with infiltration of the tissue by dendritic cells. However, there was no I-E^k expression on the bulk of Mac-1$^+$ cells infiltrating the kidneys of CBA mice, which was consistent with the findings of a predominantly PMN infiltration by conventional histology. These data suggest that responses in the kidney of CBA mice to systemic candidiasis was mediated mostly by neutrophils and that these cells were not as capable of limiting infection as the macrophages in the brain.

In contrast to the kidneys of CBA mice, inflammatory infiltrates in the kidneys of BALB/c mice were composed of a large percentage of F4/80$^+$ macrophages. Additionally, many of the cells within kidney abscesses in BALB/c mice were Ia$^+$. The pattern of inflammatory infiltration in the kidneys of BALB/c mice was similar to that seen in the brain of infected BALB/c and CBA mice. The presence of F4/80$^+$ and Ia$^+$ cells was associated with an increased resistance to infection in BALB/c mice and more rapid clearance of renal yeast load than was seen in CBA mice. Hence, these

studies demonstrated that the presence of activated macrophages was associated with increased resistance to tissue invasion in the brain. Additionally, these data suggest that increased resistance of BALB/c mice to systemic candidiasis was due, at least in part, to preferential recruitment of activated macrophages to the kidney.

The importance of macrophages was supported by the finding of increased macrophage and Ia^+ expression in the kidneys of BALB/c mice during secondary systemic candidiasis compared to primary infection. In contrast, immunised CBA mice did not have an apparent increase in Ia expression or F4/80 expression in the kidneys during systemic candidiasis and remained more sensitive to infection than naive BALB/c mice. The expression of Ia in the brain and kidneys of CBA mice would need to be confirmed with a more appropriate Ab. These studies suggest that the kidney may be the primary organ responsible for dissemination of *C. albicans* to other tissues and that control in this organ, such as was seen in BALB/c mice, is sufficient to protect from disease. This is supported by earlier observations that the kidney was the only organ to demonstrate the differences in resistance patterns between CBA and BALB/c mice at all inoculum sizes tested.

Data from other studies supports the hypothesis that macrophages are important in the resistance to and resolution of systemic candidiasis. Resistance to secondary systemic candidiasis has been linked to the activation of highly candidacidal macrophages (Bistoni *et al.*, 1988; Vecchiarelli *et al.*, 1988). The protective effects of vaccination required the continued presence of both activated macrophages and the vaccine strain of *C. albicans* (Bistoni *et al.*, 1988; Vecchiarelli *et al.*, 1988). Additionally, these effects were T-cell independent, as the results could be recapitulated in athymic mice (Bistoni *et al.*, 1988). Enhancement of macrophage function can significantly protect mice from primary systemic candidiasis (Baccarini *et al.*, 1986) and removal of macrophages decreases resistance to systemic candidiasis (Qian *et al.*, 1994). Macrophages were increased and T-cells decreased in the spleens of mice with

systemic candidiasis (Costantino, 1995a). Finally, resolution of systemic candidiais has been associated with the replacement of the predominantly PMN infiltrate in the kidneys with a mononuclear infiltrate (Romani *et al.*, 1992c; Puccetti *et al.*, 1994).

The importance of macrophages could be studied further by a more complex investigation of macrophage infiltration and the kinetics of this infiltration. Additionally, the importance of macrophages could be further assessed by macrophage depletion (administration of toxic silica or liposomes containing dichloromethyl diphosponate). The data obtained from these two experimental approaches should provide a more comprehensive evaluation of the importance of macrophages in this model of systemic candidiasis.

The role of T-cells in the resistance to primary and secondary systemic candidiasis remains elusive. $CD3^+$ cells were not found within abscesses in the brain of either BALB/c or CBA mice. $CD3^+$ cells were, however, occasionally found within the abscesses in the kidneys of both strains of mice. However, the presence of $CD3^+$ cells did not appear sufficient to protect the kidney from yeast colonisation as this organ was consistently more heavily colonised than the brain. Additionally, there was no apparent increase in the number of $CD3^+$ cells in either the brain or the kidney during secondary infection, suggesting these cells do not exert local effects on disease resistance in secondary systemic candidiasis.

The difference in susceptibility between CBA and BALB/c mice was apparent as early as day one in primary infection. This supports the hypothesis that differences in resistance phenotypes does not reside within the T-cell compartment. Many studies have shown that T-cells are important in resistance to both primary and secondary systemic candidiasis (Miyake *et al.*, 1978; Kagaya *et al.*, 1981; Sinha *et al.*, 1987; Ashman, 1990). However, in most of these studies the evidence for T-cell involvement was circumstantial (Miyake *et al.*, 1978; Kagaya *et al.*, 1981; Ashman,

1990). Additionally, other studies have demonstrated that T-cells play little role in host resistance to systemic candidiasis in either primary or secondary infection (Cutler, 1976; Rogers *et al.*, 1976; Giger *et al.*, 1978; Tabeta *et al.*, 1984; Mahanty *et al.*, 1988). Nude athymic mice are more resistant to systemic candidiasis than normal mice (Cutler, 1976). Additionally, depletion of $CD4^+$ or $CD8^+$ cells from normal mice (F1 hybrids of BALB/cCr x DBA/2Cr) had no effect on the outcome of primary systemic candidiasis (Romani *et al.*, 1992b). Hence, these studies, plus the data presented above, suggest that T-cells play little role in the resistance to systemic candidiasis in both primary and secondary systemic candidiasis.

Early resistance to *L. Monocytogenes* in SCID mice has been attributed to NK cell function (Bancroft, 1993) and it is possible these cell types could play a role in the resistance to acute systemic candidiasis. However, studies of NK cell function during systemic candidiasis in immunocompetant mice have demonstrated that they are not important for the resolution of systemic candidiasis (Romani *et al.*, 1993) and do not appear to be important contributors to the generation of Th phenotypes (Romani *et al.*, 1993). Neither do NK cells do not appear to be directly candidacidal, at least to the yeast growth form of *C. albicans.* (Baccarini *et al.*, 1983; Bistoni *et al.*, 1983; Zunino and Hudig, 1988). In addition, preliminary studies failed to find evidence of increased NK cell activity in the early stages of systemic candidiasis in CBA mice (data not shown). Consequently, it appears unlikely that NK cells play a dominant role in the early stages of the immune response to systemic candidiasis in immunocompetant mice. This also appears to be the case in immunocompetant mice during acute infection with *L. monocytogenes* (Schultheis and Kearns, 1990: Song, *et al.*, 1995: Teixeira and Kaufman 1994).

An interesting observation from these studies was that CD5 expression was often higher than $CD3^+$ expression in the kidneys of both strains of mice. CD5 is expressed on T-cells and a small sub population of B-cells. $CD5^+$ B-cells (B1 B-cells) are a sub-

population of self-renewing B-cells that may derive from a separate lineage to that of conventional B-cells. B1 cells do not have somatic rearrangement of Ig genes and have in the past been linked to autoimmunity (Kincade and Gimble, 1993). The presence of these cells in both CBA and BALB/c mice suggests a possible role for these cells in resistance to systemic candidiasis. However, the presence of these cells was not confirmed by double staining for CD5 and a B-cell marker, such as, CD45R.

In conclusion, a model of systemic candidiasis has been developed for use in this laboratory that mimics both the human disease as well as other animal models. Histological and quantitative yeast culture reveal patterns of resistance similar to those observed by others (Hector *et al.*, 1982; Ashman and Papadimitriou, 1988). In addition, these studies have been extended by the use of immunohistochemistry to establish the cell surface phenotype of the immigrant cell populations in the kidney and brain of affected mice.

Immigrant macrophages and or resident microglia were present in the brain of infected mice. In the kidneys of infected CBA mice the inflammatory infiltrate was predominantly Mac-1^{+}/Ia^{-} neutrophils. In contrast, BALB/c mice had considerable Ia and F4/80 expression in the kidney that correlated with increased resistance of this organ to infection. Inflammatory infiltrates in the brain of CBA mice and BALB/c mice and the kidneys of BALB/c mice suggest that macrophages are important in the resolution of both primary and secondary systemic candidiasis. T-cells numbers were low in the kidney and brain in both inbred strains of mice during primary and secondary systemic candidiasis. This suggests that T-cells have little effect in the resistance to systemic candidiasis, at least within the microenvironment of the brain and the kidney. Increased resistance to secondary candidiasis was apparent in BALB/c and CBA mice. However, in CBA mice, this was insufficient to compensate for the primary defect in resistance in these mice as they remained more sensitive to infection than naive BALB/c mice. This again indicates that innate responses are more

important than Ag specific responses. The lack of increased F4/80 and Ia expression in CBA mice during secondary compared to primary systemic candidiasis may explain the limited increase in resistance seen in these mice.

CHAPTER FOUR

SEMI-QUANTITATIVE PCR

Abstract

Cytokines are cell communication molecules that are produced by a range of cell types from immune cells such as T-cells, to parenchymal cells such as hepatocytes. These molecules are central to the organisation of an effective immune response and their detection and measurement are areas of considerable interest. However, with few exceptions, cytokines are produced at a very low level and often act in a paracrine or autocrine fashion. Detection of these mediators has therefore been difficult, particularly *in vivo*. The polymerase chain reaction (PCR) has made the *in vivo* detection of cytokine mRNA possible. However, the PCR has several limitations that make quantitation difficult, considerable effort is required to ensure the technique can be used for quantitation. For the purpose of this study it was necessary to develop a semi-quantitative PCR capable of measuring multiple cytokine mRNA transcripts produced in various tissues in a murine model of systemic candidiasis.

This chapter details the development of a semi-quantitative PCR that is sensitive, accurate, reproducible, and suitable for use with multiple tissue samples. The sensitivity of the assay is in the range of 2 to 20 attograms of target. Correlation coefficients of between 0.96 to 0.99 were routinely obtained when concentration of input DNA was graphed against cpm of PCR product. This technique obviates the need for the development of internal standards such as those used in quantitative techniques and therefore allows for considerable flexibility in changing the target sequence measured. The assay system is as sensitive as any of the previously described methodologies but needs considerably less optimisation and reagents. The method is the only semi-quantitative PCR the author is aware of that utilises multiplex PCR. This allows for direct comparison between different cytokine transcripts.

4.1 INTRODUCTION

Cytokines are a group of "communication" molecules central to the functioning of an effective immune system. Cytokines are general highly potent molecules that work in the pico to femtomolar range (Balkwill and Burk, 1989). Most of the cytokines, in particular lymphokines (T-cell derived), function in a paracrine and many in an autocrine fashion (Balkwill and Burk, 1989). These molecules, with the exception of the pro-inflammatory cytokines such as TNF and IL-1, are difficult to detect *in vivo*.

Cytokines are generally measured *in vitro,* by bioassay (Gillis *et al.*, 1978) or by immunoassay (Heinzel *et al.*, 1991). However, cytokine production *in vitro,* is significantly higher than that *in vivo* (Troutt *et al.*, 1992; Kelso, 1993). Consequently, bioassays or ELISA assays, except in cases of extreme pathology (Heinzel, 1990; Troutt *et al.*, 1992), are generally not sufficiently sensitive, or specific enough, to allow for the *in vivo* determination of cytokine production. Cytokine production can be monitored *in vivo* by immunohistochemistry (Jorg *et al.*, 1990), However, this method is not quantitative and some cytokines, such as IL-4, are extremely difficult to detect by immunohistochemistry.

The most effective method for the evaluation of *in vivo* cytokine production is the analysis of cytokine gene transcription, via detection of cytokine mRNA. The most sensitive method for this is RT-PCR. However, RT-PCR needs considerable optimisation to be used quantitatively (1.11).

At the commencement of this study, published quantitative methods (Becker-André and Hahlbrock, 1989; Wang *et al.*, 1989a) were not considered suitable for use with multiple tissue samples. There was a need for a semi-quantitative (SQ)-RT-PCR method that was tractable and sensitive. This chapter details the development of an alternative technique, to those previously described (Becker-André and Hahlbrock,

1989; Wang *et al.*, 1989a), more suitable for use with multiple tissue samples. The method described is sensitive, amenable to multiple sample analysis, and robust enough for the application of multiplex PCR. This method obviates the need for the development of internal standards such as those used in quantitative techniques and therefore allows for considerable flexibility in changing the target sequence measured. The assay system is as sensitive as any of the previously described methodologies. Finally, utilisation of multiplex SQ-RT-PCR allows for direct comparisons between different cytokine transcripts without the requirement for an internal control.

4.2 SUMMARY OF MATERIALS AND METHODS

4.2.1 Materials

Mice of the strains BALB/c and CBA/CaH were obtained from the Animal Resources Centre, Murdoch University, Perth, Western Australia (2.1.1).

Plasmids containing the cDNA inserts for the cytokines IL-2 (pcD Mo IL-2), IL-3 (pcD Mo IL-3), IL-4 (pcD Mo IL-4), IFNγ (pcD Mo IFNγ), GM-CSF (pcD Mo GM-CSF) and G-CSF (pMG2) were used as positive control DNA for the quantitative PCR (2.3.2). Primers used in the PCR are described in chapter 2 (2.3.1) and were chosen such that the PCR product spanned introns to allow for the differentiation of cDNA from contaminating genomic DNA.

Densitometry was carried out on a Computing Densitometer, Molecular Dynamics. Gamma (γ-) counting was performed on a Hewlett Packard Cobra II gamma counter with energy window settings of 15-1000 keV.

4.2.2 Methods

RNA extraction was performed according to the method of Chomczynski and Sacchi (1987) with modifications as described in chapter 2 (2.8.7).
The cDNA reactions were performed as described in chapter 2 (2.9.1).
Plasmid DNA (2.3.2) used as controls was purified as described in chapter 2 (2.8.5). Plasmid DNA was most commonly used in a multiplex PCR with serial dilutions of both IL-4 and IFNγ in conjunction with both sets of primer. Unless otherwise stated, standards were used as the complete plasmid not as the excised cDNA. In cases where only the cDNA insert was used, insert DNA was purified as described in chapter 2 (2.8.4).

PCR products were separated by polyacrylamide gel electrophoresis (PAGE) (2.8.1). PCR product yield was determined by densitometry or by γ-counting (2.9.5)

4.3 RESULTS

4.3.1 Optimisation of PCR

PCR optimisation was carried out for all primer sets prior to development of the SQ-PCR. Optimal $MgCl_2$ concentration was 2 or 2.5mM depending on the primer set (data not shown). All primer combinations functioned under the conditions detailed in 2.8.6.

4.3.2 PCR product quantification

During optimisation of the SQ-PCR it was necessary to determine the most accurate and sensitive method for the analysis of PCR product yield. Two of the most widely used methods for this are, β- or γ-counting of radiolabelled PCR product or densitometric analysis of autoradiographs.

4.3.2.1 Densitometric Scanning

Densitometric scanning was assessed for use in determining SQ-PCR product yield. Serial dilutions of IL-4 and IFNγ controls were subjected to SQ-PCR. The PCR product was subject to electrophoresis, and exposed to autoradiographic film. The resulting autoradiographs (Figure 4.1a and 4.1b) was used to determine band intensities by densitometric analysis. With a short exposure the two lowest dilutions

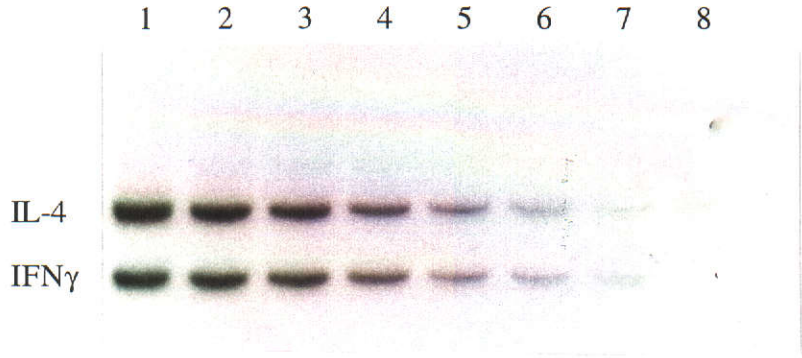

Figure 4.1a Autoradiograph of a 20 cycle IL-4/IFNγ multiplex SQ-PCR. Multiplex SQ-PCR was performed on control IL-4 and IFNγ DNA. Resultant PCR products were separated by PAGE and the gel dried. PAGE gel was exposed to x-ray film for 3 minutes and the film developed. Shown are the results of serial 1:10 dilutions of DNA, lanes 1-5 (14ng to 1.4pg) and serial 1:2 dilutions of DNA, lanes 5-8 (1.4pg to 175*fg*).

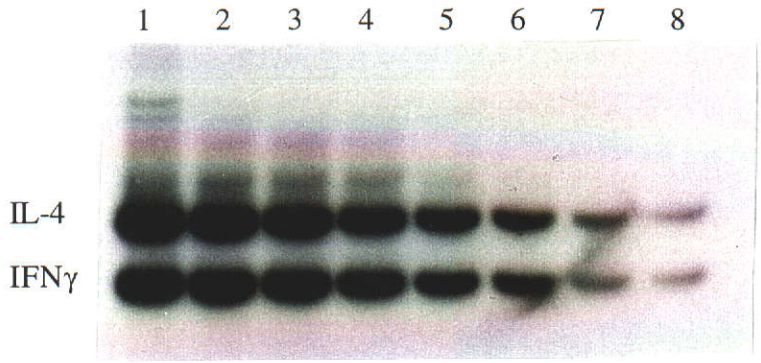

Figure 4.1b Autoradiograph of a 20 cycle IL-4/IFNγ multiplex SQ-PCR. Shown is a longer exposure time (60 minutes) of Figure 4.1a. Shown are the results of serial 1:10 dilutions of DNA, lanes 1-5 (14ng to 1.4pg) and serial 1:2 dilutions of DNA, lanes 5-8 (1.4pg to 175*fg*).

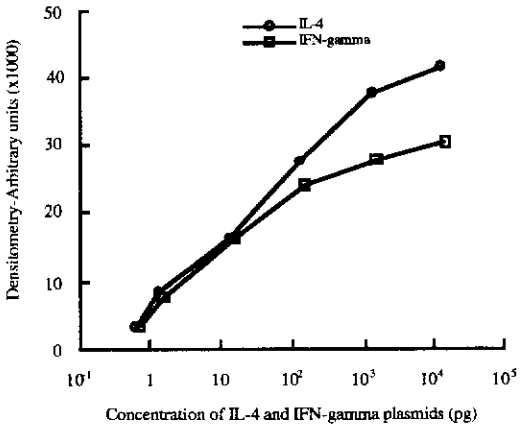

Figure 4.2 Densitometric analysis of a Figure 4.1a. Linearity was assessed by graphing band volumes against input DNA concentration from autoradiograph shown in Figure 4.1a. Linearity was evident from 1 to 1000pg of IL-4 and 1 to 100pg IFNγ plasmid control DNA.

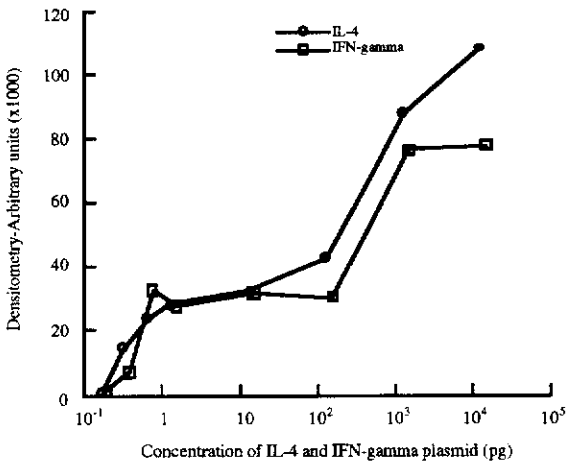

Figure 4.3 Densitometric analysis of Figure 4.1b. Linearity was assessed by graphing band volumes against input DNA concentration from autoradiograph shown in Figure 4.1b. Increased autoradiography time resulted in loss of linearity compared to that achieved with the shorter exposure time (Figure 4.2).

(lanes 7 and 8, 350 and 175fg of DNA respectively) were insufficiently exposed for densitometric analysis. A graph of concentration versus product yield produced a straight line that reached a plateau at approximately 100pg of target DNA for IFNγ and 1000pg for IL-4 (Figure 4.2). Exposure for a longer period (Figure 4.1b) increased the band intensity and allowed for densitometric determination of all the bands. However the increased exposure adversely affected the linearity of the assay (Figure 4.3).

4.3.2.2 Gamma counting

Limitations with densitometric analysis lead to a comparison between this and γ-counting. SQ-PCR was performed with serial dilutions of IL-4 and IFNγ standards from 33fg to 0.22fg IL-4 and 37.5fg to 0.29fg IFNγ respectively. Results obtained from densitometric analysis were compared to those obtained from excision of the bands and γ-counting.

The densitometer was capable of discrimination to 4.1fg and 4.7fg for IL-4 and IFNγ respectively (Table 4.1). Gamma counting allowed for the discrimination of results to 1.0fg and 1.1fg for IL-4 and IFNγ respectively (Table 4.1). Standard curves were produced from the values given in Table 4.1 (data not shown). These standard curves were used to determine the concentration of test DNA included in the assay. Input concentration of IL-4 was 6.1fg, input concentration of IFNγ was 7.0fg. When γ-counting was used to produce a standard curve, values obtained for IL-4 and IFNγ tests were 6.7fg and 8.3fg, respectively. When densitometry was used, the IL-4 test sample was unreadable and the value obtained for IFNγ was 9.7fg. Therefore, γ-counting was both more sensitive and more accurate than densitometry.

Standard (concentration *fg*)	Densitometry band volume	γ-counting (cpm)
IL-4 (32.8)	300	3163
IL-4 (16.4)	100	1893
IL-4 (8.2)	0	417
IL-4 (4.1)	62	830
IL-4 (2.0)	0	121
IL-4 (1.0)	0	26
IL-4 (0.5)	0	7.5
IL-4 (0.2)	0	24
IFNγ (37.5)	292	2885
IFNγ (18.7)	132	1627
IFNγ (9.3)	0	385
IFNγ (4.6)	82	592
IFNγ (2.3)	0	171
IFNγ (1.1)	0	44
IFNγ (0.6)	0	18
IFNγ (0.3)	0	24

Table 4.1 Comparison of γ-counting and densitometry. Results of a multiplex SQ-PCR on plasmid DNA containing IL-4 and IFNγ cDNA inserts. Shown is a comparison of the sensitivity of γ-counting compared to densitometry.

4.3.3 [α-^{32}P] dCTP incorporation

To maximise specific activity of the PCR product whilst maintaining sensitivity the PCR was carried out with constant amounts of [α^{32}p] dCTP (3000Ci/mM) titrated against serial 1 in 10 dilutions of dCTP. In this experiment, RNA was extracted from the spleen of CBA mice four days post infection with $1x10^6$ viable *C. albicans* blastopores. Total RNA was used in a RT-SQ-PCR with the exception that the amount of dCTP added was titrated from 83μM to 0.083μM dCTP. PCR was performed for 25 cycles with each titration in duplicate. Control DNA was included at a concentration of 2*f*g IL-4 and IFNγ with two dilutions of dCTP, 8.3μM and 0.83μM.

Decreasing the concentration of dCTP increased the specific activity of PCR product (data not shown). However, the increased specific activity of the product appeared to be at the expense of sensitivity, as reduced sensitivity was apparent at lower dilutions of dCTP when control DNA was amplified (data not shown). To test this, serial dilutions of plasmid controls were used in a SQ-PCR with the dilutions of dCTP detailed above.

PCR was performed with serial 1/2 dilutions of IL-4 and IFNγ plasmids (4.4*f*g to 0.275*f*g respectively) and serial 1/10 dilutions of dCTP (from 83μM to 0.083μM). PCR product could be detected from 4.4 to 1.1*f*g of IL-4 and IFNγ using 83μM dCTP (lanes 1-3 Figure 4.4). With 8.3μM dCTP, product could be detected from 4.4 to 0.27*f*g of IL-4 and IFNγ (Lanes 6-10 Figure 4.4). This was an increase in sensitivity at least four fold greater than was achieved when 83μM dCTP was used. Hence, the sensitivity of the assay was improved by increasing the specific activity of the PCR product. When 0.83μM dCTP was used in the SQ-PCR, DNA was only detectable at the lowest dilution of 4.4*f*g (lane 11 Figure 4.4). When 0.083μM dCTP was used in the SQ-PCR, IL-4 and IFNγ plasmid was completely undetectable at the

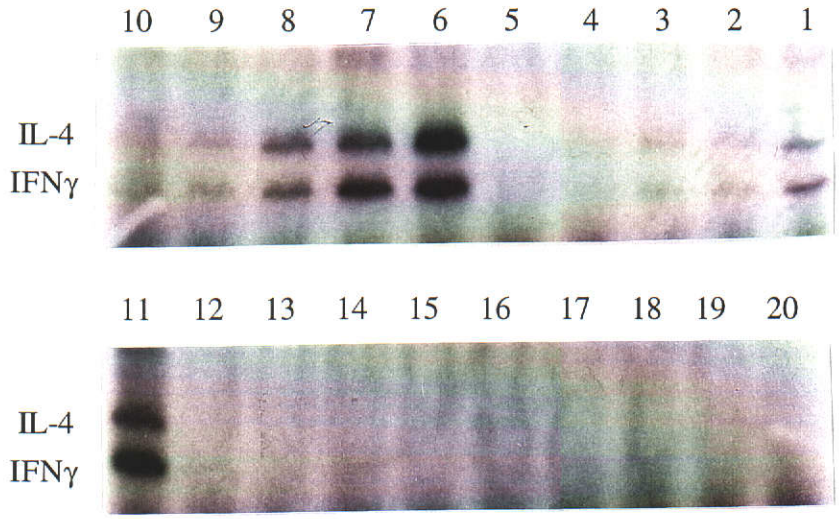

Figure 4.4 Effect of "cold" dCTP concentration on multiplex SQ-PCR sensitivity. To determine if decreased "cold" dCTP increased the specific activity of the PCR product at the expense of sensitivity, serial dilutions of IL-4 and IFNγ standards were amplified by SQ-PCR in the presence of serial dilutions of "cold dCTP. Lanes 1-5 show the PCR product for IL-4 and IFNγ from serial 1/2 dilutions of input DNA (4.4 to 0.27*f*g) following SQ-PCR using 83μM dCTP. Lanes 6-10 show the PCR product for IL-4 and IFNγ from serial 1/2 dilutions of input DNA (4.4 to 0.27*f*g) following SQ-PCR using 8.3μM dCTP. Lanes 11-15 show the PCR product for IL-4 and IFNγ from serial 1/2 dilutions of input DNA (4.4 to 0.27*f*g) following SQ-PCR using 0.83μM dCTP. Lanes 16-20 show the PCR product for IL-4 and IFNγ from serial 1/2 dilutions of input DNA (4.4 to 0.27*f*g) following SQ-PCR using 0.083μM dCTP. Maximal sensitivity was achieved with 8.3μM dCTP seen in lanes 6-10.

concentrations used (lanes 16-20 Figure 4.4). Therefore, optimal sensitivity and specific activity was achieved with a dCTP concentration of 8.3μM.

4.3.4 β-actin semi-quantitative PCR

The sensitivity required for β-actin SQ-PCR was considerably lower than was required for cytokine detection (Data not shown). As a result, β-actin PCR was carried out with increased concentration of dCTP (16.7μM) and decreased radiolabelled [α^{32}p] dCTP (0.014pM) as described (2.9.3).

4.3.5 Sensitivity

Multiplex SQ-PCR was performed on serial dilutions of plasmids containing IL-4 and IFNγ cDNA to determine sensitivity of the assay (Figure 4.5). IL-4 and IFNγ products were verified by size and Sanger sequencing (data not shown). Specific PCR product bands were visible to 0.12*f*g for IL-4 and 0.15*f*g IFNγ (Lane 7 Figure 4.5). Repeats of this experiment gave sensitivities in the range of 0.12 to 0.51*f*g (Figure 4.6 and data not shown). This correlated to a sensitivity of approximately 30 to 122 molecules. When IL-4 insert cDNA was excised from plasmid control and used for PCR, detection was in the range of 2 to 20 attograms (data not shown). This indicated that a levels of sensitivity could be achieved in the order of 4 to 44 molecules of target DNA. Previous data had shown the assay was accurate to at least 6*f*g of target sequence (4.3.2.2).

SQ-PCR was performed on duplicate, 1*f*g samples of control DNA for IL-2, IL-3, IL-4, IFNγ, G-CSF and GM-CSF to ensure that amplification efficiencies and accuracy were similar for a range of cytokine mRNA species. Variations in the yield of IL-2, IL-3, IL-4, IFNγ, and GM-CSF were within 1 standard deviation of the mean yield.

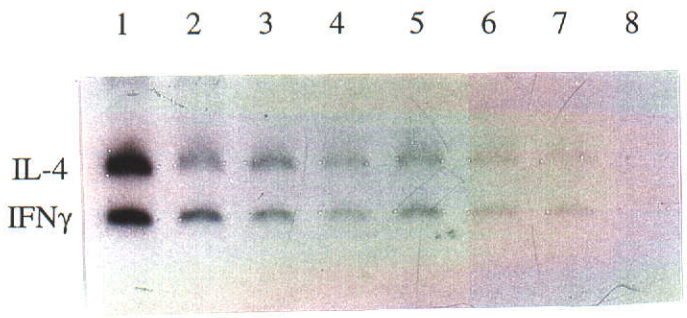

Figure 4.5a Multiplex SQ-PCR on serial dilutions of IL-4 and IFNγ control DNA. Lanes 1 to 7 are 1:2 dilutions of IL-4 from $8fg$ to $0.12fg$ and IFNγ 9 to $0.15fg$. Lane 8 is a negative control.

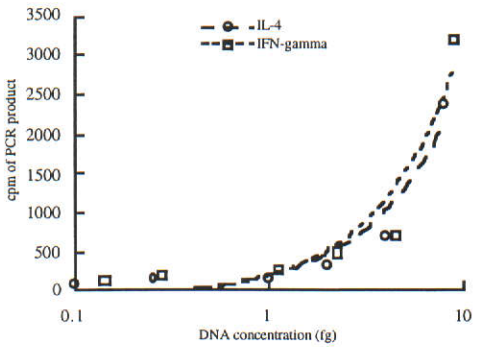

Figure 4.5b Standard curve produced from γ-counting of bands excised from Figure 4.5a. Linearity of the assay was reduced below approximately $1fg$.

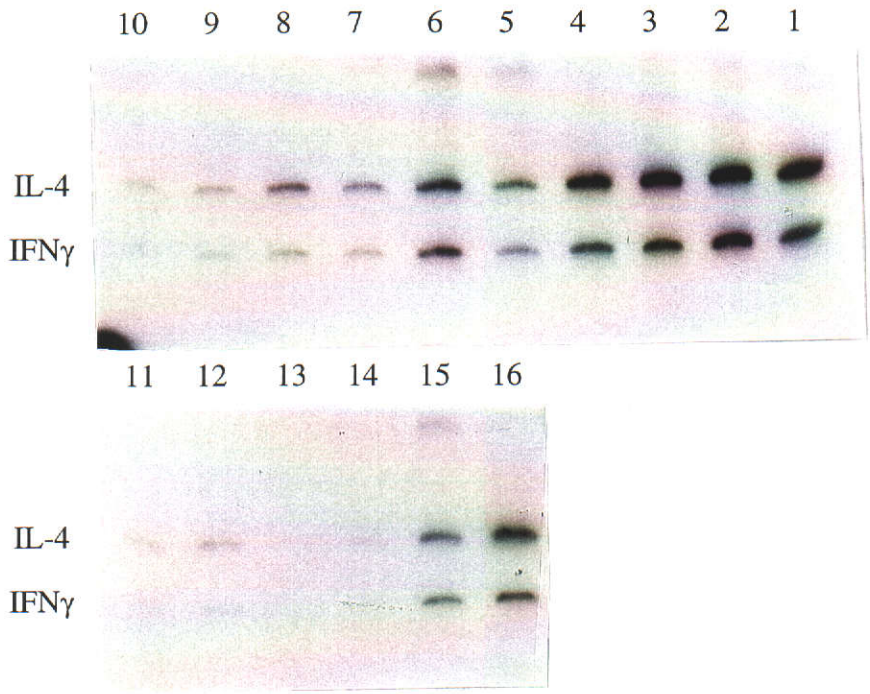

Figure 4.6 Multiplex SQ-PCR (25 cycle) on IL-4 and IFNγ. Total rat RNA was added to plasmid DNA to determine if the addition of RNA had an effect on the sensitivity, accuracy or linearity of SQ-PCR. Shown is the autoradiograph of IL-4/IFNγ SQ-PCR following a 1:2 dilution of plasmid from $32fg$ to $1fg$ IL-4 and $36fg$ to $1.2fg$ IFNγ from lanes 1 to 14 respectively, each dilution was performed in duplicate. Lanes 15 and 16 represent duplicate dilutions of "unknowns" of IL-4 and IFNγ. The addition of total rat RNA had no effect on linearity (correlation co-efficients for IL-4 and IFNγ were 0.96 and 0.97 respectively) or sensitivity (both IL-4 and IFNγ were detectable to 1 and $1.2fg$ respectively) or accuracy of multiplex SQ-PCR. Standard curves drawn from these results were used to estimate the DNA concentration of test samples. Estimated IL-4 concentration was $9fg$, estimated IFNγ concentration was $20fg$, input concentrations were $10fg$ and $12fg$ respectively.

PCR product yield for G-CSF was within 2 standard deviations of the mean (data not shown).

It was considered possible that the heterogenous mix of moieties contained in a cDNA sample, obtained from total tissue RNA, could contain contaminating species that would affect quantitation by RT-SQ-PCR. Therefore, total rat liver RNA and/or rat liver cDNA was added to plasmid DNA. The addition of rat liver cDNA or RNA had no effect on the sensitivity of the RT-SQ-PCR (Figure 4.6 and data not shown).

4.3.6 Linearity

Using the reaction conditions described (2.9.2), serial dilutions of plasmids containing IL-4 and IFNγ cDNA were co-amplified to determine linearity of the assay. Bands were excised from the gels, counted and the resultant cpm graphed against input concentration. Standard curves produced in this manner consistently had correlation co-efficients of between 0.96 to 0.99 (Figure 4.5b and data not shown).

Linearity, and hence accuracy, of the assay was reduced below approximately $1fg$ of plasmid DNA (Figure 4.5b). For a 25 cycle PCR the lower level of linearity correlated to approximately 240 molecules whilst linearity remained to at least 7900 molecules (data not shown). For a 20 cycle PCR linearity was maintained up to 240 million molecules for IL-4 and 20 million molecules for IFNγ (Figure 4.2). Linearity was unaffected by the addition of rat liver cDNA (Figure 4.6).

4.3.7 Correcting for Total mRNA

β-actin primers were included in multiplex RT-SQ-PCR for the cytokines IL-4 and IFNγ. Following a 25 cycle multiplex RT-SQ-PCR little or no IL-4 or IFNγ product was visible on an over night autoradiograph (Figure 4.7). In contrast, β-actin bands were intense. A longer autoradiography exposure time allowed for the positioning of faint cytokine bands but inhibited the positioning of intense β-actin bands. To overcome this problem β-actin primer concentrations were reduced 50 (final concentration 4nM) and 100 (final concentration 2nM) fold to attenuate the β-actin signal (Figure 4.8). No bands for β-actin were visible in any of the duplicate samples, although IL-4 and IFNγ were visible in duplicates (lanes 1-4 and 6-9 Figure 4.8). This allowed for the determination of cytokine mRNA product otherwise not detected (Figure 4.7 and Figure 4.8). A 10 fold reduction in β-actin primer concentration did not significantly alter β-actin product yield (data not shown). Dilutions of β-actin primers from 1/20 and 1/30 did reduce product yield, whilst maintaining detection of β-actin. However, β-actin PCR duplicates were unacceptably variable when β-actin primers were diluted below 1/20 (data not shown).

Consistent results demonstrated that the addition of β-actin primers to multiplex RT-SQ-PCR inhibited the cytokine determination, such that duplicates were unacceptably variable (data not shown). It was noted throughout the development of the RT-SQ-PCR that if the amount of β-actin message (and therefore mRNA) differed substantially from sample to sample, then normalisation to β-actin was adversely affected. This often resulted in artificially raising the cytokine ratio to β-actin in samples with low β-actin, with the reverse occurring in samples with high β-actin or total mRNA.

To overcome the effect of β-actin inhibition of cytokine PCR and variable mRNA yields two approaches were adopted. In the first approach the amount of oligo-dT

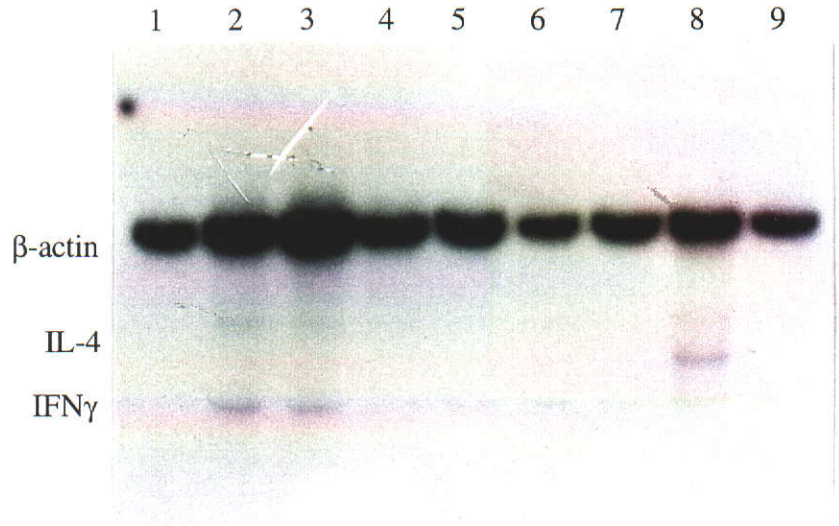

Figure 4.7 Multiplex RT-SQ-PCR on splenic mRNA with β-actin, IL-4 and IFNγ primers. β-actin mRNA bands were visible in all samples. However, IL-4 mRNA was faintly detectable in lanes 2, 3 and 8 only. IFNγ mRNA was faintly detectable in lanes 2, 3, 5, 6, 7, 8 and 9.

used in the cDNA reaction was reduced to allow competition to occur for the oligo-dT primer. This should ensure, that, given excess total mRNA, each sample has a similar yield of cDNA due to limited oligo-dT concentration. In a series of experiments cDNA was produced with serial 1/2 dilutions of oligo-dT primer. No effect was seen in yield of β-actin following SQ-PCR on cDNA produced with dilutions of oligo-dT ranging from 1μM to 61pM (Data not shown). Dilutions lower than this did result in reduced signal (Data not shown). However, the β-actin results failed to reflect oligo-dT dilution (Data not shown).

The second approach was to perform the RNA extraction in a manner that was as consistent as possible. This involved extracting RNA from a constant amount (50mg) of tissue in an unvarying fashion. This "quantitatively" extracted RNA was then used in an RT reaction and subjected to SQ-PCR for β-actin message. Shown in Figure 4.9 (lanes 1-7) are the results of β-actin RT-SQ-PCR on 7 independent RNA samples extracted from the brain of CBA and BALB/c mice. The results indicate that quantitative extraction of RNA can be used to ensure total mRNA yields from multiple tissue samples are consistent. Consequently, all subsequent samples were extracted "quantitatively".

The results of SQ-RT-PCR for β-actin mRNA were used to produce a normalisation value (2.9.6) that was used to correct cytokine SQ-RT-PCR values to the total mRNA concentration. During the course of this study it became apparent that the method devised for the quantitative extraction of total RNA was extremely reproducible. In view of this, in later experiments the normalisation values were not used to correct the cytokine data.

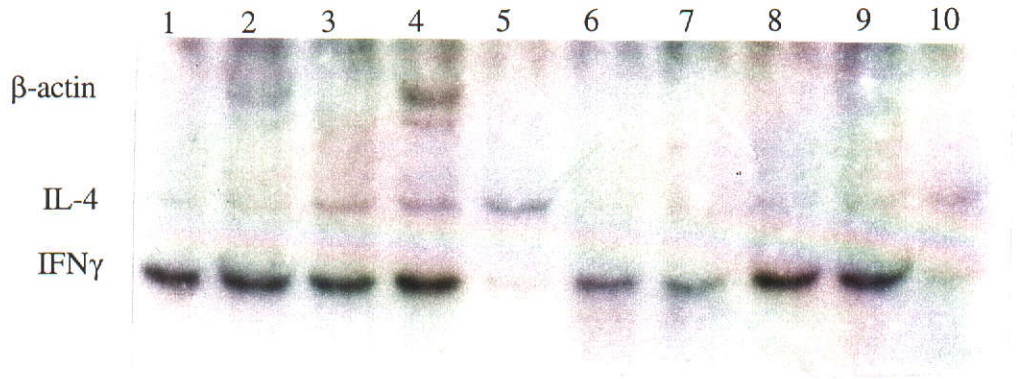

Figure 4.8 Attenuation of β-actin PCR product by dilution of β-primers. RT-SQ-PCR on duplicate splenic mRNA samples with β-actin, IL-4 and IFNγ primers. β-actin primer concentrations in lanes 1-4 was 4nM (1:50), β-actin primer concentrations in lanes 6-9 was 2nM (1:100). Lanes 5 and 10 show PCR products from positive control DNA (IL-4 and IFNγ at 2 and 2.5*fg* respectively). β-actin bands were only faintly detectable in lanes 2 and 4 with a 1:50 dilution of β-actin primers. In contrast β-actin was not detectable in the same samples when amplified using β-actin primers at 2nM . IL-4 and IFNγ was detectable in all samples. This was in contrast to that seen in Figure 4.7. PCR products seen in lanes 1, 2, 6 and 7 were amplified from the same sample as was used in lane 1 in Figure 4.11. PCR products seen in lanes 3, 4, 8 and 9 were from the same sample as was used in lane 2 in Figure 4.7.

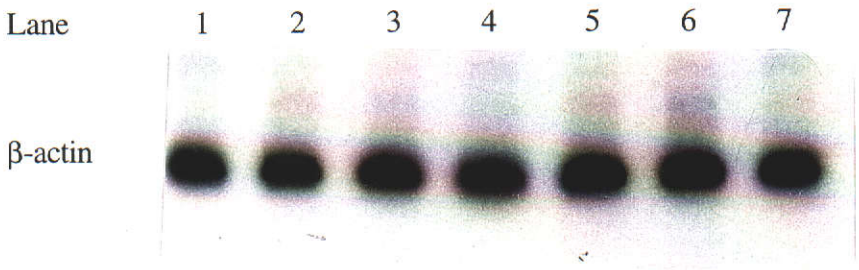

Figure 4.9 Quantitative extraction of total RNA from the brains of 7 individual mice. RNA was extracted in a "quantitative" fashion and the amount of β-actin message was assessed by RT-SQ-PCR as a measure of total mRNA. Autoradiograph shows that mRNA can be extracted in a "quantitative" fashion.

4.3.8 Multiplex PCR

To minimise sample numbers and to allow for comparison of different cytokines mRNA species within the same sample, multiplex RT-SQ-PCR was employed. Multiple cytokine primer pairs could be added to the same SQ-PCR tube providing the PCR products were of a dissimilar size. The maximum number of primer pairs used was five. Figure 4.10 shows a representative multiplex RT-SQ-PCR of RNA extracted from the spleen of uninfected BALB/c and CBA mice. Multiplex PCR of this sample showed high TNFα mRNA expression, moderate levels of IL-4 and IFNγ mRNA, minimal IL-2 mRNA and no IL-3 mRNA. In initial experiments the results of multiplex PCR were confirmed by single RT-SQ-PCR (data not shown). This was later found to be unnecessary as the PCR product yields obtained from multiplex RT-SQ-PCR were similar to those obtained after conventional RT-SQ-PCR. However, absence of a particular cytokine mRNA was confirmed by single RT-SQ-PCR to ensure that amplification efficiencies were not compromised by competition from other primer sets.

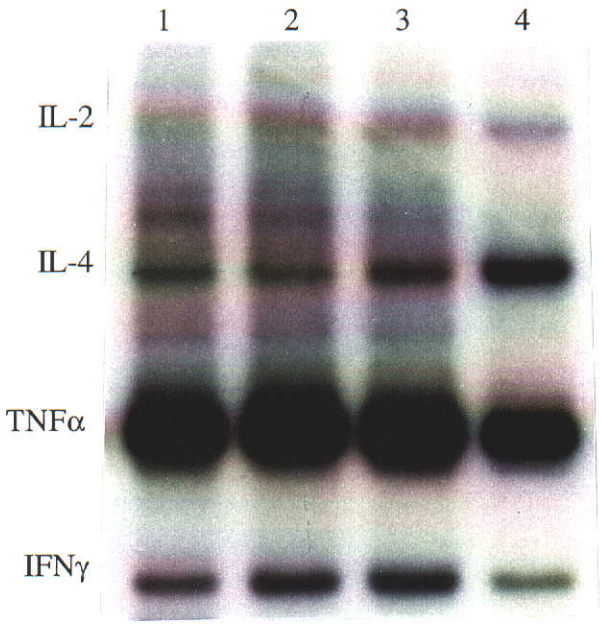

Figure 4.10 Multiplex RT-SQ-PCR on total splenic RNA for IL-2, IL-3, IL-4, TNFα and IFNγ cytokine gene expression. Lanes 1 and 2 are from uninfected BALB/c mice, lanes 3 and 4 are from uninfected CBA mice. IL-3 was below the limit of detection in the spleens of uninfected mice.

4.4 DISCUSSION

Cytokines are a group of "communication" molecules that are central to the organisation of an effective immune response. Consequently there is considerable interest in their expression during the development of the immune response. However, the measurement of cytokines *in vivo* is complicated by their low and transient expression coupled with a short half life. Conventional techniques, such as bioassays, are not sufficiently sensitive for this measurement. The advent of PCR has greatly facilitated this analysis (Wang *et al.*, 1989a; Gilliland *et al.*, 1990).

In this study cDNA and plasmid control DNA was used to develop a multiplex RT-SQ-PCR method for the *in vivo* detection of cytokine gene expression. The method was developed to circumvent many of the problems associated with quantitative PCR techniques (1.11) as well as to produce a method uniquely suited to the analysis of multiple samples and cytokines.

The first essential of the SQ-PCR was to determine the most accurate method for measuring PCR product yield. Other studies have employed various methods for determining PCR product yield (Rappolee *et al.*, 1989; Gilliland *et al.*, 1990; Murphy *et al.*, 1990; Lagoo-Deenadaylan *et al.*, 1993). In this assay direct incorporation of [α^{32}p] dCTP during PCR was chosen over end labelling of PCR primers so as to increase the specific activity of PCR product (Eeles and Stamps, 1993). Gamma or scintillation counting was chosen over densitometric analysis as it was more sensitive and accurate. The sensitivity of densitometry could be increased by lengthening the exposure time of autoradiography. However, increasing the exposure time adversely affected the linearity of the assay. Similar findings have been reported by others (Murphy *et al.*, 1990).

The low concentration of cytokine mRNA production *in vivo* required maximal sensitivity. To this end it was necessary to optimise [α^{32}p] dCTP incorporation. This was achieved by reducing the concentration of dCTP, while maintaining [α^{32}p] dCTP concentration. Increased incorporation of [α^{32}p] dCTP resulted in increased specific activity of the PCR product. However, this was accompanied by a reduction in the sensitivity of the assay. Reducing dCTP by a factor of 20 compared to the other dNTP's, produced the optimal balance of increased specific activity and sensitivity. For the assay of β-actin mRNA there was no requirement for increased sensitivity. Consequently β-actin PCR was carried out with increased concentration of dCTP (16.7μM) and decreased [α^{32}p] dCTP (0.014pM).

The lower limit of sensitivity has not been reported for many of the quantitative PCR techniques (Chelly *et al.*, 1988; Wang *et al.*, 1989a; Gilliland *et al.*, 1990). Those that do report lower levels of sensitivity record sensitivities in the range of 100 (Becker-André and Hahlbrock, 1989) to ~3700 molecules (Funk and FitzGerald, 1991). Sensitivity for the PCR in repeat experiments using plasmid DNA with a cDNA insert was in the order of 30 and 122 molecules. Sensitivity using excised cDNA was in the range of 2-22 molecules. Therefore, the sensitivity of the RT-SQ-PCR assay developed was equal to or better than other described methods.

Standard curves (PCR product yield vs input DNA concentration) produced using the SQ-PCR method developed typically yielded correlation coefficients of between 0.96 to 0.99. Test samples included into the PCR demonstrated the assay was capable of determining mRNA concentration in a quantitative fashion. The linear range of the assay varied with the number of cycles used. With a 20 cycle PCR, the assay was linear from at least 240 000 molecules up to 240 million molecules for IL-4 and up to 20 million molecules for IFNγ. For a 25 cycle PCR the lower limit of linearity correlated to approximately 240 molecules whilst linearity remained to at least 7900 molecules. Hence, the linearity and accuracy of the assay was in the same range as the

technique described by Becker-André and Hahlbrock (1989). Finally, contaminating species such as RNA and cDNA had little effect on the SQ-PCR.

If direct incorporation of [α^{32}p] dCTP is used as a mechanism of detecting PCR product yield, then the cpm of PCR product will be governed by the final yield of the product and the number of cytidine residues in the initial target. While many synthetic internal standards techniques correct for the difference in cytidine residues in the targets (Funk and FitzGerald, 1991), other do not (Kramnick *et al.*, 1993). Correction for cytidine residues is not required with the endogenous standard techniques as each sample is compared to the same endogenous standard and the technique makes no attempt to be quantitative. However, when comparing different cytokines with similar amplification efficiencies, such as IL-4 and IFNγ, in a multiplex PCR the number of cytidine residues in each target maybe influence quantitation. However, for the purposes of this study no correction factor was employed as it was not clear how the increase in number of dCTP residues affected the degree of incorporation. This did not appear to affect quantitation as dilutions of control plasmid for different cytokines gave consistently similar incorporation of [α^{32}p] dCTP, even though product size and number of cytidine residues varied.

Standard curves were not produced for all the cytokines assayed in this study. However, to ensure the sensitivity of detection was similar for each cytokine, 1*f*g of IL-2, IL-3, IL-4, IFNγ, G-CSF and GM-CSF control plasmids were subject to SQ-PCR. All cytokine controls (with the exception of G-CSF) gave results less than 1 standard deviation from the mean. G-CSF cDNA control was between 1-2 standard deviations from the mean. Controls were not available for TNFα and IL-1α.

For SQ-PCR it is necessary to control for various "tube effects" by the use of an endogenous standard (Chelly *et al.*, 1988; Luqmani *et al.*, 1992; Babu, 1993; Iizawa *et al.*, 1993). This allows for a comparison of test sequence, such as a cytokine mRNA,

to a internal standard sequence. This compensates for any inter tube variations as each test is compared to a standard that has undergone the same treatment.

In the development of this SQ-PCR, β-actin signal was consistently higher than that of the target sequence. The differential level of mRNA expression lead to difficulties in monitoring cytokine expression, as β-actin bands were frequently over exposed. This complication has been reported for other SQ-PCR methodologies (Kellogg and Kwok, 1990). Attempts to attenuate the β-actin signal by reducing primer concentration were not successful and lead to loss of signal or decreased reproducibility. Increased variability at low β-actin primer concentrations, was probably due to premature entry into the plateau phase due to changes in target/primer ratio. The amplification of β-actin along with cytokine cDNA appeared to set up competition for reaction components, such as dNTPs and Taq, with the major target (β-actin) which reduced the signal of the cytokine message. This effect of competition by the endogenous standard has been demonstrated by others (Murphy *et al.*, 1990).

Due to the competitive effects of the β-actin primers, other methods of controlling for total mRNA concentration and "tube effects" were attempted. Serial dilutions of oligo-dT were used in the RT reaction in an attempt to limit total cDNA concentration as a function of input oligo-dT. This methodology was unsuccessful as dilutions of oligo-dT less than 61pM resulted in considerable variation in the PCR product that did not reflect concentration of oligo-dT. Use of oligo-dT concentrations above 61pM had no effect on PCR product yield.

Another approach taken to ensure that each sample had similar levels of total mRNA was to quantitatively extract total RNA. It was found that RNA could be "quantitatively" extracted. Quantitative extraction was verified by β-actin SQ-PCR. This "quantitative" extraction has been demonstrated by others (Lagoo-Deenadaylan *et al.*, 1993) and was used to ensure total mRNA concentrations were similar in all RNA

samples. This does not, however, allow for the control of inter-tube variation in amplification efficiencies However, due to the competition seen when β-actin was included in cytokine PCR it was not feasible to use the endogenous standard in the same tube. Consequently RNA was extracted in a "quantitative" fashion and the total mRNA concentration was assessed by SQ-PCR of β-actin. To minimise tube effects each sample was analysed for cytokine mRNA concentration in duplicate. Each sample was given a normalisation value (2.9.6). This allowed the cytokine mRNA concentrations to be normalised to total mRNA concentration as determined by β-actin RT-SQ-PCR. In later experiments this was found to be unnecessary.

The RT-SQ-PCR so far described is not quantitative, in that, it was not designed to give absolute numbers of cytokine molecules per gram of tissue and no attempt was made in this direction. Instead the technique was designed to be semi-quantitative, in that, it allowed for the comparison of cytokine mRNA production between samples. The application of multiplex PCR allowed for the comparisons of different cytokine mRNA species within the same sample. Quantitative techniques so far described have been extremely labour intensive as well as expensive (Becker-André and Hahlbrock, 1989; Wang *et al.*, 1989a; Gilliland *et al.*, 1990). Further, there are several inherent problems in assuming the quantitative techniques that are based on constructed controls are accurate. Many standards are large cytokine multi-constructs (Reiner *et al.*, 1993) and hence molarity of the construct must be accounted for. Standards are often dsDNA which can have different amplification efficiencies to that of single stranded cDNA (Carding *et al.*, 1992). Differences in size between standard and target may introduce differences in the amplification efficiency. However, Gilliland *et al* (1990) found no such differences in efficiency. Competition has been demonstrated between the internal control and the target sequence in the internal standard assay (Babu, 1993). Others have found such competition to be minimal (Kramnick *et al.*, 1993). Finally, results from each of the quantitative methodologies need to be corrected for total mRNA concentration. This necessitates use of an internal standard

such as β-actin which introduces many of the errors associated with semi-quantitative techniques.

In summary, an SQ-PCR technique has been devised with a sensitivity in the range of 4 to 44 molecules that is linear from approximately 240 to at least 7900 molecules. The technique relies on optimised incorporation of [α^{32}p] dCTP and measurement of product by scintillation or γ-counting. Whilst the technique does not have an internal control, tube effects are minimised by measuring each sample in duplicate. Similar total mRNA concentrations in each sample was ensured by "quantitative" extraction of RNA. Intern, the "quantitative" extraction of the RNA was assessed by semi-quantitative β-actin PCR. The method has been adapted for multiplex PCR of up to 5 cytokine sets and requires minimal optimisation when new target sequences are measured. Finally, the method is optimal for measuring cytokines in many tissues when a comparative study is required.

CHAPTER FIVE

CYTOKINE mRNA PROFILES OF THE SPLEEN

Abstract

The inbred strains of mice, BALB/c and CBA, have different susceptibilities to systemic candidiasis, with CBA mice more susceptible to infection than BALB/c mice. Cytokine mRNA expression in the spleens of BALB/c and CBA mice was assessed using RT-SQ-PCR during the acute phase of systemic candidiasis to determine if differences in susceptibility could be linked to the expression of cytokine genes. There was no evidence of a Th1 response in BALB/c mice, with both IL-4 and IFNγ mRNA species produced at similar levels. A similar early response was seen in CBA mice, however these was a later pattern of IL-4 and IFNγ mRNA production that was consistent with a Th2 response. The shift to a Th2 like pattern of cytokine mRNA expression in the CBA mice was coincident with increased mortality in these mice. However, the shift in cytokine mRNA profiles was detected after initial differences in sensitivities to infection had become apparent. This suggests the change in cytokine profile was a sequelae to a failure to resolve the infection in CBA mice. Analysis of the mRNA for cytokines that increase the candidacidal activity of phagocytic cells did not reveal evidence to suggest a deficiency in the production of these cytokines in CBA mice. PGE_2 has been demonstrated to enhance the generation of a Th2 phenotype, however, inhibition of PGE_2 did not protect CBA mice from systemic candidiasis. Conversely, inhibition of PGE_2 production increased the sensitivity of CBA mice to chronic systemic candidiasis and reduced the expression of IFNγ mRNA. Inhibition of PGE_2 production also increased the sensitivity of BALB/c mice to acute systemic candidiasis. In direct contradiction, administration of neutralising mAb to IFNγ protected CBA and BALB/c mice from acute systemic candidiasis. In conclusion, there was no evidence from cytokine profiles of the spleen to account for the differential susceptibility of CBA and BALB/c mice to acute systemic candidiasis. However, there was a later response in CBA mice consistent with expression of a Th2 phenotype in the spleen of these mice. This Th2 pattern of cytokine mRNA production was coincident with increased mortality of CBA mice and could not be reversed by inhibition of PGE_2 production. Contradictory data on the expression of IFNγ and neutralisation of IFNγ suggests this cytokine may have different roles in the acute infection compared to chronic infection.

5.1 INTRODUCTION

Cytokines are a group of "communication" molecules produced by both lymphoid and non-lymphoid cells that are integral to the organisation of an effective immune response. Hence, the profile of cytokines produced during infection may be a guide to the type of ensuing immune reaction. In 1986 Mosmann and colleagues described the presence of two subsets of Th cells from long term murine T-cell clones, which differed in the patterns of cytokines they secreted. Mosmann and colleges described these cells as Th1 cells on the basis of IL-2, IFNγ and TNFβ secretion. Th2 cells were categorised on the basis of IL-4 and IL-5 secretion. Both cell types secreted IL-3, TNFα and GM-CSF. The cytokine patterns produced correlate to cell mediated/DTH responses for Th1 and humoral and allergic responses for Th2. Therefore, increased IFNγ may indicate the presence of a Th1 response or cell mediated response, whereas high levels of IL-4 may be indicative of a Th2, or humoral response.

Since the initial classification of T-helper responses into Th1 or Th2 by Mosman *et al* (1986), the Th1 subset has been extensively linked to disease resistance in animal models. The archetypal disease model this has been applied to is leishmaniasis. In this disease, *Leishmania major* produces cutaneous leishmaniasis, the resolution of which is dependant on the ensuing immune response. Sensitive or non healer mice, in this case BALB/c, produce a typical Th2 response to infection (Scott *et al.*, 1988; Heinzel *et al.*, 1989). Healer mice, such as C57Bl/6 or C3H/HeN, produce a typical Th1 response (Howard, 1986; Scott *et al.*, 1988; Heinzel *et al.*, 1989). The injection of anti-IFNγ mAb in the early phase of the disease can reverse the development of a Th1 response in healer mice producing a non-healer Th2 phenotype (Belosevic *et al.*, 1989). The reciprocal experiment with anti-IL-4 mAb, in non-healer mice induces a protective Th1 response (Sadick *et al.*, 1990). The monocyte derived cytokine IL-12 can also drive a curative Th1 response (Heinzel *et al.*, 1993; Sypek *et al.*, 1993). In

contrast to the leishmaniasis model, Th2 phenotypes are protective in certain nematode infections (Lange *et al.*, 1994). Additionally the Th1 phenotype has been linked to disease exacerbation in autoimmunity, where the Th1 response is responsible for excessive immunopathology (Scott *et al.*, 1994)

In models of systemic candidiasis the Th1/Th2 paradigm has been used to explain differences in disease susceptibility in inbred strains of mice and F1 hybrids of inbred strains of mice (Romani *et al.*, 1991b; Romani *et al.*, 1992a; Romani *et al.*, 1992c; Romani *et al.*, 1993). However, it is not clear that this paradigm fits all models of systemic candidiasis. Additionally, early experiments with the same strains of mice and yeast used in some of the above models, suggested that T-cells played little or no role in the increased resistance to secondary infection (Bistoni *et al.*, 1988; Vecchiarelli *et al.*, 1988). Furthermore, there is ample evidence to suggest that cytokines other than those involved in the Th1/Th2 paradigm are involved in the resolution of a range of diseases (Reiner, 1987; Cillari *et al.*, 1989; Nakane *et al.*, 1989; Roll *et al.*, 1990) including systemic candidiasis (Van't Wout *et al.*, 1988; Kullberg *et al.*, 1990; Cenci *et al.*, 1991; Allendoerfer *et al.*, 1993),

There is considerable evidence that *C. albicans* has an immunosuppressive effect on cell mediated immune responses (Marmor and Barnett, 1968; Canales *et al.*, 1969; Paterson *et al.*, 1971; Laforce *et al.*, 1975; Twomey, 1975), possibly due to *C. albicans* mannan (MAN) or MAN metabolites (Fisher *et al.*, 1978; Podzorski *et al.*, 1990). MAN may induce immunosuppression by stimulating the inappropriate release of excess PGE_2 from macrophages (Witkin *et al.*, 1986; Nelson *et al.*, 1991). PGE_2 is a powerful, macrophage released, immunomodulator that is capable of inhibiting IL-12 release from macrophages (van der Pouw Kraan *et al.*, 1995) and may contribute to immunosuppression (Witkin *et al.*, 1983; Witkin *et al.*, 1986; Nelson *et al.*, 1991) or immune deviation to a Th2 response (Roper *et al.*, 1990; Betz and Fox, 1991; Snijdewint *et al.*, 1993).

This chapter describes the measurement of a range of cytokine mRNA species produced in the spleen of mice during systemic candidiasis. The cytokines mRNA species IFNγ and IL-4 as representative of the Th1 and Th2 subsets respectively and other cytokines mRNA species to gauge responses that may not be reflected by a typical Th1/Th2 pattern. The effect of PGE_2 on systemic candidiasis was also investigated by use of the cyclooxygenase inhibitor indomethacin. IFNγ was inhibited by neutralising mAb to determine if enhanced expression of this cytokine contributed to the increased resistance to BALB/c mice compared to CBA mice.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Mice of the strains BALB/c and CBA/CaH were obtained from the Animal Resources Centre, Murdoch University, Perth, Western Australia (2.1.1)

PCR primers used for SQ-RT-PCR are described in chapter 2 (2.3.1) and were chosen such that the PCR product spanned introns, allowing for the discrimination of cDNA from contaminating genomic DNA. PCR cDNA standards used in this study are described in chapter 2 (2.3.2).

The IFNγ neutralising mAb HB-170 (from the hybridoma RA-642) was a kind gift from Dr. L. Harrison, Walter and Elisa Hall Institute of Medical Research. HB-170 was provided as an ascites produced preparation at 1mg/ml.

5.2.2 Methods

Mice were inoculated with viable *C. albicans* as described in chapter 2 (2.5.2)
RNA was extracted as described in chapter 2 (2.8.7)
cDNA synthesis and RT-SQ-PCR was performed as described in chapter 2 (2.9)
Neutralisation of IFNγ was achieved by administration of 0.5mg HB-170 mAb/mouse *iv* 24 hours prior to *iv* inoculation of *C. albicans* blastospores.
Indomethacin inhibition of the cyclooxygenase pathway was performed as described in chapter 2 (2.5.4)
Quantitative yeast culture was performed as described in chapter 2 (2.5.3).
Statistical analyses were performed by the Mann-Whitney U test.

5.3 RESULTS

5.3.1 Intra splenic IL-4 and IFNγ mRNA production during systemic candidiasis

To determine the level of IL-4 and IFNγ mRNA production in the spleens of infected mice, $1x10^6$ *C. albicans* blastospores were injected *iv* into healer BALB/c and non healer CBA mice. Control mice received sterile non-pyrogenic saline. Splenic IL-4 and IFNγ mRNA levels were assessed in total RNA extracted from the spleens of infected and uninfected mice over the first seven days of infection by RT-SQ-PCR. Plasmids containing the cDNA inserts for IL-4 and IFNγ were used as controls. Multiplex SQ-PCR of control IL-4 and IFNγ plasmids produced a ratio of IL-4 to IFNγ PCR product of 1.7:1 (data not shown). This indicates a slight increase in the amplification efficiency of IL-4 to IFNγ and/or increased incorporation of [α^{32}p] dCTP into the larger IL-4 product.

5.3.1.1 *In vivo* IL-4 mRNA production

Uninfected BALB/c mice produced substantial amounts of IL-4 mRNA with an average cpm of the PCR product over 3000 (Figure 5.1a). In contrast, there was an immediate reduction in IL-4 mRNA production following infection in BALB/c mice with IL-4 mRNA levels over 7 fold lower 24 hours following infection than in uninfected mice. Following day one there was a greater than 4 fold increase in IL-4 mRNA levels to day three. Following a peak at day three, the level of IL-4 mRNA decreased by over 3 fold in infected BALB/c mice over the ensuing period to day seven (Figure 5.1a). Similar results were obtained in at least one repeat SQ-PCR using the same total mRNA (data not shown).

Uninfected CBA mice had similar levels of IL-4 mRNA to those seen in uninfected BALB/c mice (Figure 5.1a and b). Following infection in CBA mice, there was an

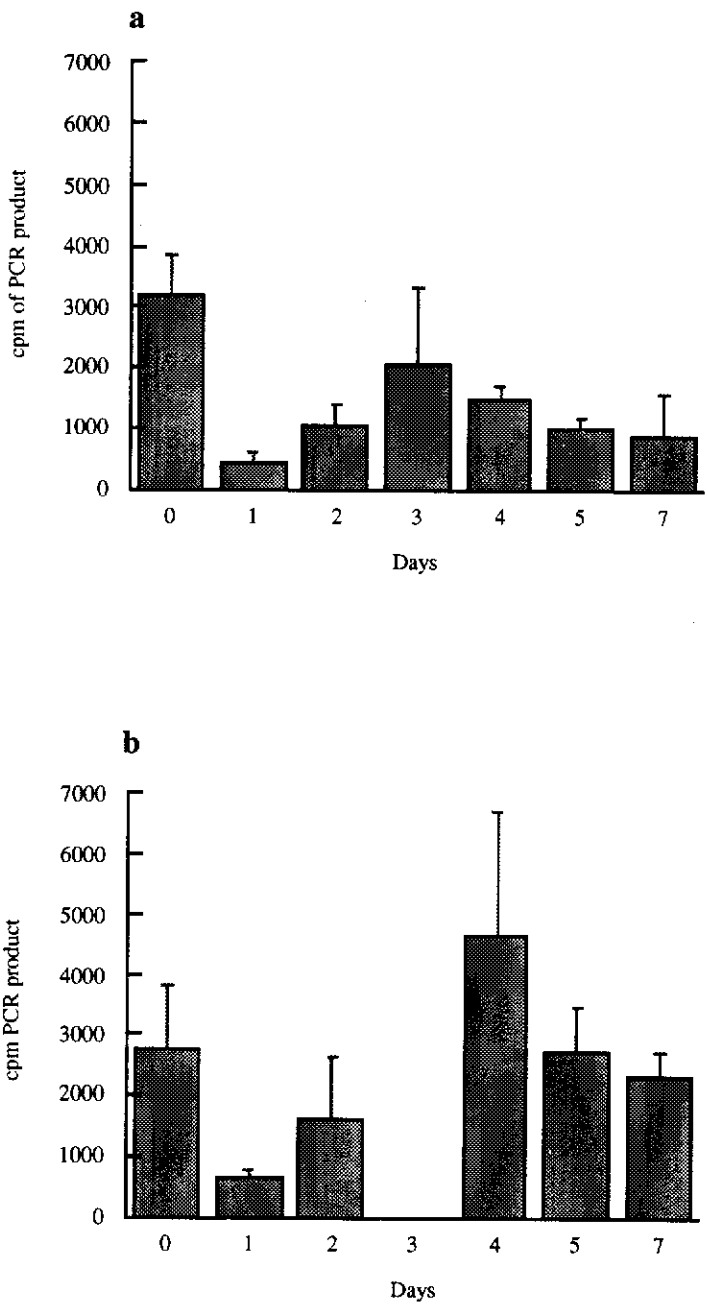

Figure 5.1 *In vivo* IL-4 mRNA production in the spleens of BALB/c (a) and CBA (b) mice, following *iv* inoculation of $1x10^6$ viable. *albicans* blastospores. RNA was extracted and subjected to multiplex RT-SQ-PCR for the quantitation of IL-4 and IFNγ mRNA. Values for day zero represent the mean ± SEM for five uninfected mice. Values for day one to five represent mean ± SEM for four infected mice. Values for day seven represent mean ± SEM for three infected mice. IFNγ mRNA production is shown in Figure 5.2.

initial drop in IL-4 mRNA levels at days one and two, which were 4 and 1.7 fold lower than in uninfected mice respectively. By day four, however, IL-4 mRNA levels in infected CBA mice were higher than uninfected CBA mice. Repeat experiments showed levels of IL-4 mRNA were similar to, or slightly higher, on day three in comparison to day four in CBA mice (data not shown). Following day four, levels of IL-4 mRNA in infected CBA mice remained similar to those of uninfected mice. Similar results were obtained in at least one repeat RT-SQ-PCR using the same total mRNA (data not shown).

5.3.1.2 *In vivo* IFNγ mRNA production

In infected BALB/c mice there was a 2 fold reduction in the amount of IFNγ mRNA produced on day one compared to uninfected mice (Figure 3.2a). IFNγ mRNA expression levels were subsequently increased from days two to four post infection in infected BALB/c mice. IFNγ mRNA expression over this period was similar to that of uninfected mice. From a peak at day three, levels of IFNγ mRNA in infected BALB/c mice declined by 3 fold over the ensuing period to day seven.

The inhibition of IFNγ mRNA seen in the first 24 hours of infection in BALB/c mice was not demonstrated in infected CBA mice (Figure 3.2b). Peak levels of IFNγ mRNA were detected in infected CBA mice at day four. From a peak at day four, there was a 5 fold reduction in IFNγ mRNA to day five and a 10 fold reduction to day seven. Repeat experiments showed levels of IFNγ mRNA were similar to, or slightly higher, on day three in comparison to day four in CBA mice (data not shown).

Production of IFNγ mRNA was consistently lower in both strains of mice compared to that of IL-4 mRNA. This trend was seen in up to five independent experiments using the same total RNA. When the amount of IL-4 mRNA was expressed as a ratio to

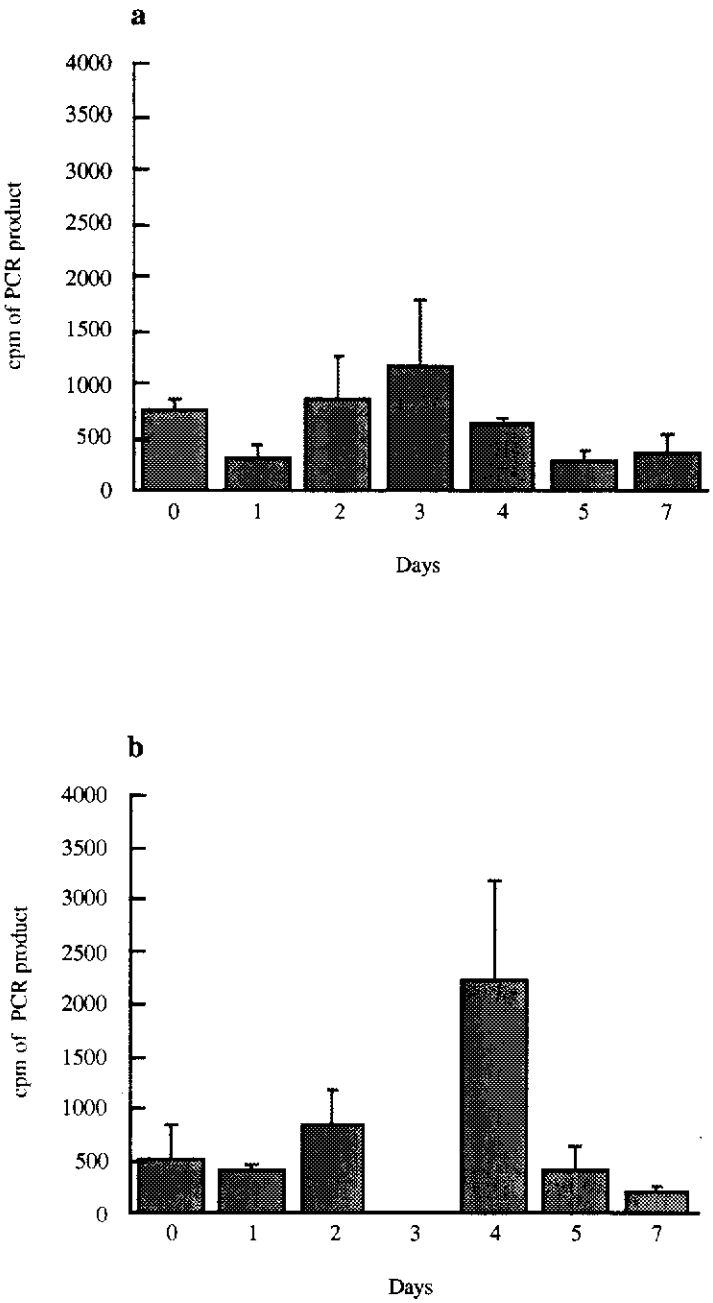

Figure 5.2 *In vivo* IFNγ mRNA production in the spleens of BALB/c (a) and CBA (b) mice, following *iv* inoculation of $1x10^6$ viable *C. albicans* blastospores. RNA was extracted and subjected to multiplex RT-SQ-PCR for the quantitation of IL-4 and IFNγ mRNA. Values for day zero represent the mean ± SEM for five uninfected mice. Values for day one to five represent mean ± SEM for four infected mice. Values for day seven represent mean ± SEM for three infected mice. IL-4 mRNA production is shown in Figure 5.1.

IFNγ mRNA (Table 5.1) uninfected BALB/c and CBA mice produced 4.2 and 5.1 fold more IL-4 mRNA than IFNγ mRNA respectively. However, in the first two days of infection this ratio was reduced in both strains of mice, up to 3.5 fold. This indicated a relative increase in IFNγ mRNA compared to IL-4 mRNA in the acute stages of infection. In the BALB/c mice this reduced IL-4/IFNγ mRNA ratio remained low during the course of the infection, with a slight increase toward levels found in uninfected mice by day five and seven. In contrast, CBA mice maintained the lowered ratio of IL-4 to IFNγ mRNA to day four only. The IL-4/IFNγ mRNA ratio increased in infected CBA from day five and seven to, 6 and 11 respectively (Table 5.1). This was evident predominantly because of the significant reduction in IFNγ mRNA production in the spleen of CBA mice over this period.

5.3.2 Intrasplenic IL-2, IL-3 and TNFα mRNA production during systemic candidiasis

The RNA and cDNA samples used in 5.3.1 were subjected to multiplex RT-SQ-PCR to determine splenic IL-2, IL-3 and TNFα mRNA production during systemic candidiasis.

5.3.2.1 *In vivo* IL-2 mRNA production

Multiplex SQ-RT-PCR for IL-2, IL-3 and TNFα mRNA was performed on total splenic RNA. IL-2 mRNA production was at the limit of detection for the RT-SQ-PCR (data not shown). Following infection in BALB/c mice there was a reduction in the amount of IL-2 mRNA produced in the BALB/c mice for the first three to four days compared to uninfected mice. However, there was considerable fluctuation in the IL-2 mRNA levels, likely due to low levels of production. The CBA mice evidenced a similar reduction in IL-2 mRNA levels in the early stages of the infection. However,

	Splenic IL-4/IFNγ mRNA ratio	
Day	BALB/c	CBA
0	4:1	5:1
1	1:1	2:1
2	1:1	2:1
3	2:1	ND
4	2:1	2:1
5	4:1	6:1
7	3:1	11:1

Table 5.1 Splenic IL-4/IFNγ mRNA ratios in the spleens of BALB/c and CBA mice. Mice were challenged *iv* with $1x10^6$ *C. albicans* blastospores and the spleen tissue removed on the days indicated. RNA was extracted and subjected to RT-SQ-PCR. Values shown are the ratio of IL-4 mRNA to IFNγ mRNA produced during the course of infection. Ratio was achieved by dividing mean IL-4 mRNA values by mean IFNγ mRNA values. Standards for IL-4 and IFNγ subjected to the same multiplex PCR gave a ratio of 1.7:1.

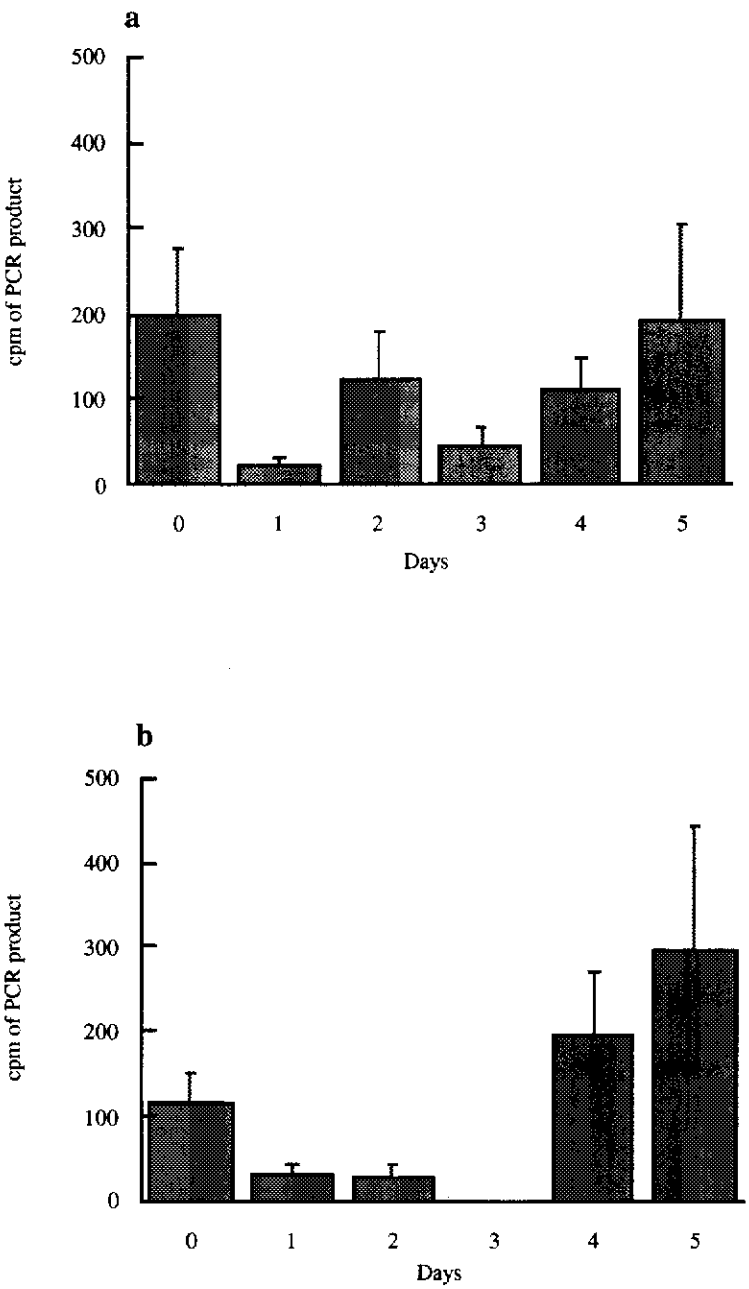

Figure 5.3 *In vivo* IL-2 mRNA production in the spleens of BALB/c (a) and CBA (b) mice, following *iv* inoculation of $1x10^6$ viable C. *albicans* blastospores. RNA was extracted and subjected to multiplex RT-SQ-PCR for the quantitation of IL-2, IL-3 and TNFα mRNA. Values for day zero represent the mean ± SEM for five uninfected mice. Values for day one to five represent mean ± SEM for four infected mice.

levels recovered by day four and had increased above those seen in control mice by day five, which was over 2 fold higher than in uninfected mice.

5.3.2.2 *In vivo* IL-3 mRNA production

No IL-3 mRNA was detected in any splenic RNA samples from either uninfected or infected mice.

5.3.2.3 *In vivo* TNFα mRNA production

TNFα mRNA production was reduced in both strains of mice during the course of the infection (Figure 5.4). BALB/c mice had a substantial reduction in TNFα mRNA in the infected mice compared to controls. The reduction of TNFα mRNA in infected BALB/c mice was constant over the course of the infection and ranged from 3 to 8.5 fold lower than in uninfected mice. A similar initial reduction in TNFα mRNA expression was seen in infected CBA mice, with an approximate reduction in the level of TNFα mRNA expression of 6 fold compared to uninfected CBA mice. However, in contrast to BALB/c mice, the levels of TNFα mRNA increased during the course of the infection in CBA mice, such that by day seven, TNFα mRNA production in infected mice was similar to uninfected mice.

5.3.3 Intrasplenic IL-1α and GM-CSF mRNA production during systemic candidiasis

The RNA and cDNA samples used in 5.3.1 were subjected to multiplex RT-SQ-PCR to determine splenic IL-1α and GM-CSF mRNA production during systemic candidiasis.

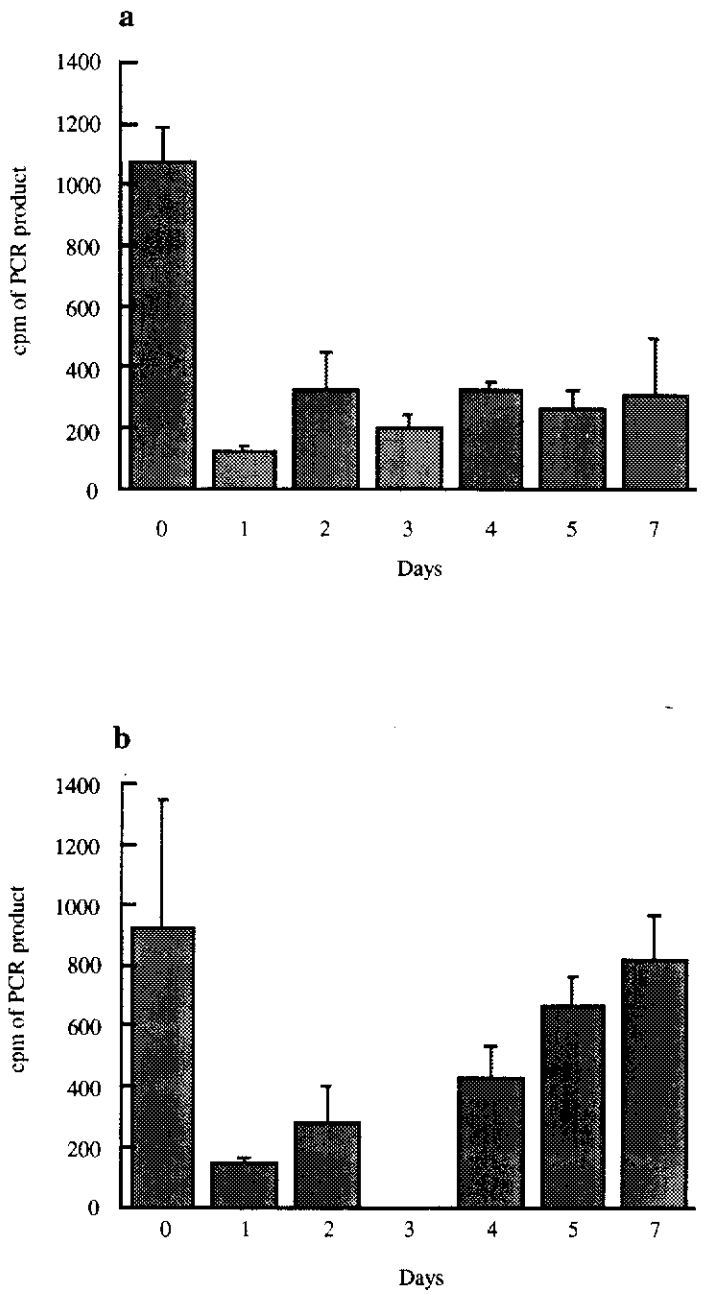

Figure 5.4 *In vivo* TNFα mRNA production in the spleens of BALB/c (a) and CBA (b), following *iv* inoculation of $1x10^6$ viable C. *albicans* blastospores. RNA was extracted and subjected to multiplex RT-SQ-PCR for the quantitation of IL-2, IL-3 and TNFα mRNA. Values for day zero represent the mean ± SEM for five uninfected mice. Values for day one to five represent mean ± SEM for four infected mice. Values for day seven represent mean ± SEM for three infected mice. IL-2 and IL-3 mRNA species were not detectable.

5.3.3.1 *In vivo* IL-1α mRNA production

Infected BALB/c mice had suppressed IL-1α mRNA production in relation to uninfected mice over the three days tested, one, four and seven. Infected BALB/c mice had IL-1α mRNA levels as assessed by RT-SQ-PCR 2.9 to 6.6 fold lower than uninfected BALB/c mice. In contrast, infected CBA mice evidenced suppression of IL-1α mRNA production only on day four, with a 7 fold reduction over that seen in uninfected CBA mice. Levels of IL-1α mRNA in infected CBA mice were similar to uninfected CBA mice on days one and seven post inoculation.

5.3.3.2 *In vivo* GM-CSF mRNA production

No GM-CSF mRNA was detected in any splenic RNA samples from either uninfected or infected mice (data not shown). Additionally, no GM-CSF mRNA was detected when the sensitivity of the assay was increased by use of a conventional 40 cycle PCR (data not shown). Additionally, GM-CSF mRNA was not detected in spleen tissue in any of three independent experiments using conventional 40 cycle PCR (Data not shown).

5.3.4 Intra splenic macrophage- (M)-CSF mRNA production during systemic candidiasis

RT-SQ-PCR was used to determine M-CSF mRNA production in the spleens of mice during systemic candidiasis. Levels of M-CSF in the spleen of infected BALB/c mice were 2, 3.5 and 4.8 fold lower than in the spleens of uninfected BALB/c mice on days one, four and seven post infection, respectively (Figure 5.6a). In contrast CBA mice have no evident suppression of M-CSF production and had increased M-CSF mRNA production on days one and four compared to uninfected CBA mice and similar levels to uninfected CBA mice on day seven (Figure 5.6b).

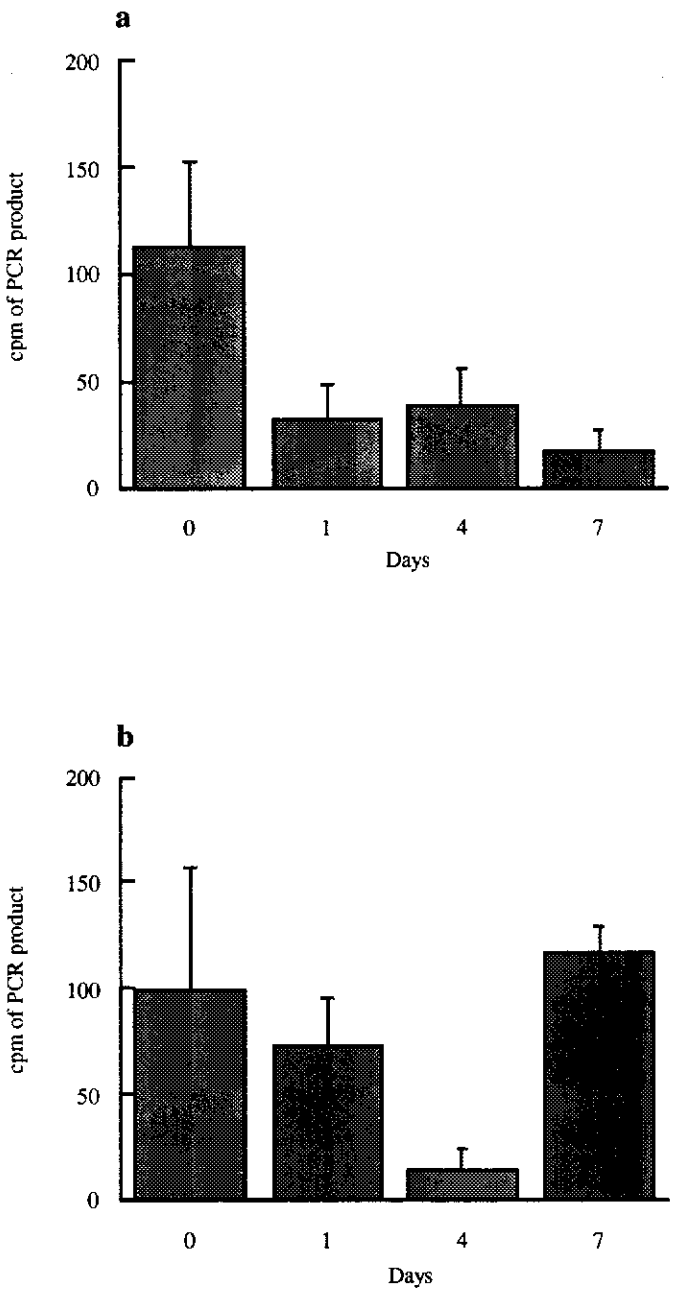

Figure 5.5 *In vivo* IL-1α mRNA production in the spleens of BALB/c (a) and CBA (b) mice, following *iv* inoculation with $1x10^6$ viable *C. albicans* blastospores. RNA was extracted and subjected to RT-SQ-PCR for quantitation of IL-1α mRNA. Values for day zero represent the mean ± SEM for five uninfected mice. Values for days one and four represent mean ± SEM for four infected mice. Values for day seven represent mean ± SEM for three infected mice.

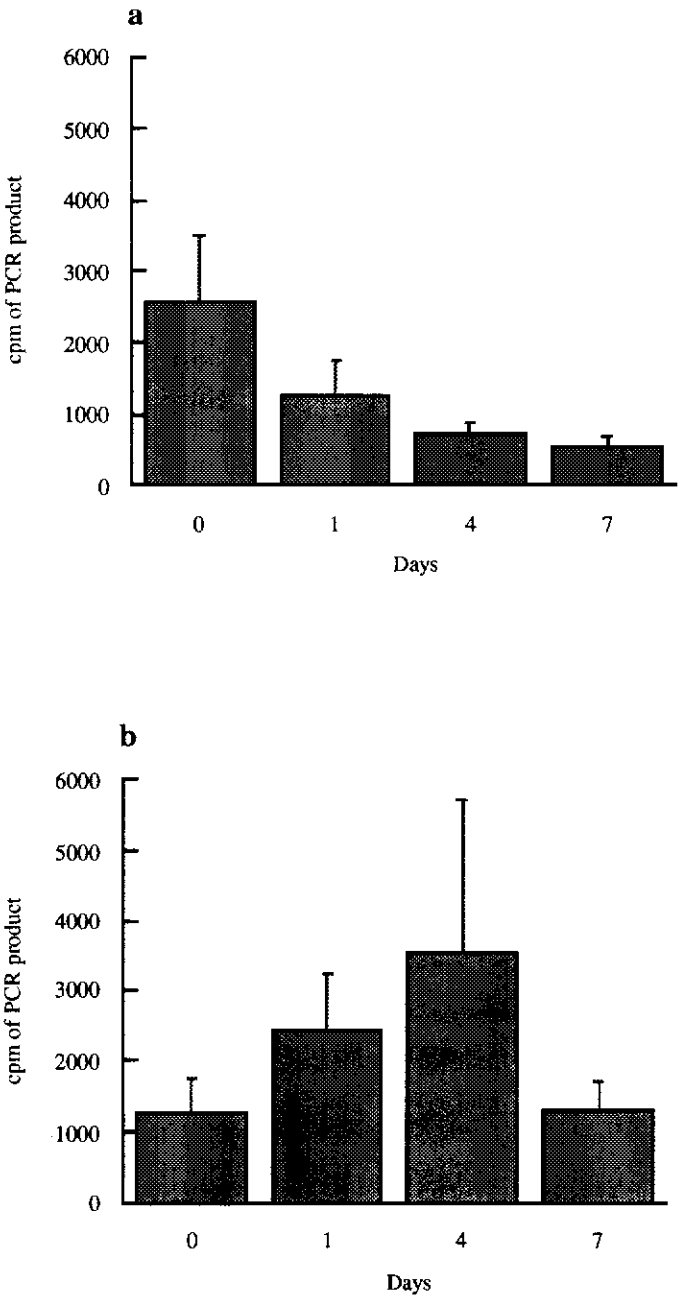

Figure 5.6 *In vivo* M-CSF mRNA production in the spleens of BALB/c (a) and CBA (b) mice, following *iv* inoculation with $1x10^6$ viable *C. albicans* blastospores. RNA was extracted and subjected to RT-SQ-PCR for quantitation of M-CSF mRNA. Values for day zero represent the mean ± SEM for five uninfected mice. Values for days one and four represent mean ± SEM for four infected mice. Values for day seven represent mean ± SEM for three infected mice.

5.3.5 Intra splenic granulocyte- (G)-CSF mRNA production during systemic candidiasis

G-CSF mRNA was not be detected in either strain of mouse, either infected or uninfected, by RT-SQ-PCR (Data not shown). Additionally, G-CSF mRNA was undetectable using conventional 40 cycle PCR in any of three independent experiments.

5.3.6 Inhibition of IFNγ

Several studies have demonstrated that resistance to bacterial and yeast infection can be mediated by Th1 cytokines, presumably due, in the most part to the effect of IFNγ on phagocytic cells such as macrophages. IFNγ is the major cytokine activator of macrophages. To test this hypothesis, BALB/c mice were inoculated with the IFNγ neutralising mAb HB-170, 24 hours prior to *iv* inoculation of viable *C. albicans* blastospores. Isotype matched control Ab was not used as it is becoming common not to use such controls (Fong *et al.*, 1989; Sadick *et al.*, 1990; Allendoerfer *et al.*, 1993) and specifically it has been demonstrated during systemic candidiasis, in both IFNγ and IL-10 ablation experiments, that data obtained using control Ab was similar to that obtained with saline alone (Romani *et al.*, 1992b; Romani *et al.*, 1994) .

Preliminary experiments suggested mice were protected from systemic candidiasis by neutralisation of IFNγ. To test this further, CBA and BALB/c mice were inoculated *iv* with $3x10^5$ viable *C. albicans* blastospores, 24 hours after receiving 0.5mg of anti-IFNγ Ab in sterile non-pyrogenic saline *iv*. Mice were sacrificed on day three to assess the effects of IFNγ depletion on acute primary systemic candidiasis. Kidneys were removed and the number of *C. albicans* enumerated by quantitative organ culture. IFNγ depleted mice were compared to mice treated 24 hours prior to *C. albicans* inoculation with non-pyrogenic saline.

	Mice	
Treatment	BALB/c mice	CBA mice
Saline	5.7 ± 0.2	7.0 ± 0.3
mAb HB-170	4.5 ± 0.3*	5.7 ± 0.1*

Table 5.2 The effect of anti-IFNγ treatment on *C. albicans* colonisation levels in the kidneys of BALB/c and CBA mice during primary systemic candidiasis. Data shown are the mean ± standard deviation (n=3-5) of tissue colonisation (log_{10} cfu/g kidney) three days after inoculation (*iv*) of $3x10^5$ viable *C. albicans*. Asterisk denotes significant reduction in tissue colonisation in mAb treated compared to saline treated groups of mice.

Results from day three quantitative yeast culture are shown in Table 5.2. Both CBA and BALB/c mice showed significant increases in resistance to infection following neutralisation of IFNγ with mAb HB-170. BALB/c mice treated with HB-170 had a 15 fold reduction in yeast load in the kidneys compared to control mice. HB-170 treated CBA mice had a 22 fold reduction in yeast load compared to saline treated controls.

5.3.7 Inhibition of PGE_2 production by inhibition of the cyclooxygenase pathway

Consistent demonstration of reduced cytokine production during systemic candidiasis indicated that generalised immunosuppression may be induced by *C. albicans*. Immunosuppression of this type may be due to increased production of mediators other than cytokines such as corticosteroids or PGE_2. Additionally, the increased production of IL-4 mRNA and decreased production of IFNγ mRNA in CBA mice after day four post infection may be due to the action of PGE_2. To test these hypotheses, mice were treated with indomethacin, an inhibitor of the cyclooxygenase pathway and hence PGE_2 production. The effects of PGE_2 inhibition was monitored in acute infection in CBA mice and BALB/c mice by quantitative organ culture and in chronic infection in CBA mice by mortality. Levels of cytokine mRNA were measured by multiplex RT-SQ-PCR to determine if indomethacin treatment increased the level of cytokine mRNA or increased the ratio of IFNγ to IL-4 mRNA.

5.3.7.1 Inhibition of the cyclooxygenase pathway during acute murine systemic candidiasis.

CBA and BALB/c mice were inoculated with $3.7x10^5$ viable *C. albicans* blastospores *iv* one hour after receiving 20μg of indomethacin *iv* to determine if inhibition of PGE_2 production protected mice from systemic candidiasis. Mice were given a further 20μg of indomethacin (*iv*) 10 hours after infection and then twice daily. Control mice were

	Mice	
Treatment	BALB/c	CBA
Iv ethanol	5.03 ± 0.5	6.7 ± 0.2
Iv indomethacin	5.8 ± 0.3§	6.7 ± 0.3

Table 5.3 The effect of indomethacin treatment on the resistance of BALB/c and CBA mice to primary systemic candidiasis. CBA and BALB/c mice were given 20μg indomethacin *iv* or 0.4% ethanol. Mice were challenged one hour later with $3.7x10^5$ viable *C. albicans* blastospores, with a further 20μg indomethacin or 0.4% ethanol within 10 hours. Mice then received 20μg indomethacin or ethanol, *iv*, twice daily. All mice were sacrificed on day three and kidneys removed for quantitative yeast culture to assess yeast load. Values given are the mean and standard deviation of $\log_{10}$ cfu/g kidney (n=5).

§ Tissue colonisation was significantly higher in indomethacin treated BALB/c mice compared to ethanol treated BALB/c mice. No significant difference was seen in the yeast load in the kidneys of CBA mice when treated with indomethacin compared to ethanol.

inoculated with PBS plus 0.4% ethanol and $3.7x10^5$ viable *C. albicans* blastospores *iv*. Mice were sacrificed on day three and their kidneys removed for quantitative yeast culture.

There was no significant difference in the yeast load in the kidney of CBA mice receiving indomethacin in comparison to mice receiving ethanol only (Table 7.3). This was repeated in 3 of 4 experiments, when indomethacin was given either orally or intravenously or in an increased dosage of 100µg/injection. In only one experiment did indomethacin protect CBA mice as assessed by quantitative organ culture on day two (data not shown). In contrast, BALB/c mice treated with indomethacin had significantly higher levels of yeast load in the kidneys compared to BALB/c mice receiving ethanol only (Table 3.2). This was repeated in two further experiments, one of which used 100µg indomethacin/injection (data not shown). BALB/c mice were more sensitive to the toxic effects of indomethacin than CBA mice and had considerable GIT stasis following treatment with of 100µg/injection indomethacin. GIT stasis in BALB/c mice was presumably due to inhibition of smooth muscle contraction as a result of inhibition of prostaglandin synthesis.

5.3.7.2 Inhibition of the cyclooxygenase pathway in CBA mice during chronic systemic candidiasis.

It was considered possible that PGE_2 has its major effect on the cell mediated arm of the immune response to systemic candidiasis. Therefore, the effects of indomethacin treatment were studied in the chronic infection when cell mediated responses were likely to be active. CBA mice were used in this study as BALB/c mice were less tolerant to indomethacin treatment. Twenty CBA mice were given $3x10^5$ viable *C. albicans* blastospores *iv*, half were given drinking water with 30µg/ml indomethacin and the other half given drinking water with 0.1% ethanol. Mortality was assessed in 7/10 mice from each group (Figure 5.8). The remaining three mice

from each group were sacrificed on day ten for quantitative yeast culture and determination of cytokine mRNA production.

Indomethacin had no protective effect on resistance to systemic candidiasis in CBA mice (Figure 5.8), indeed indomethacin treatment exacerbated disease. Mice given indomethacin in their drinking water succumbed to the infection four days earlier than CBA mice that received 0.1% ethanol only. Mice given indomethacin had 100% mortality over eighteen days, the control group had 57% mortality.

There was no significant difference in the recovery of yeast from either the kidney or the brain of indomethacin or ethanol treated mice. Yeast load in the indomethacin treated mice was 6.2 ± 0.78 $\log_{10}$ cfu/kidney and 4.14 ± 0.38 $\log_{10}$ cfu/g brain at day ten. Ethanol treated controls had 5.8 ± 0.64 $\log_{10}$ cfu/g kidney and 3.9 ± 0.18 $\log_{10}$ cfu/g brain. Similar levels of yeast were found in the kidneys of ethanol treated mice on day nineteen compared to day ten (6.14 ± 0.32 $\log_{10}$ cfu/g). In contrast, yeast load was 50 fold lower in the brain of ethanol treated mice at day nineteen compared to that seen in ethanol treated mice at day ten (2.2 ± 1.1 $\log_{10}$ cfu/g).

5.3.7.3 *In vivo* IL-4, IFNγ and TNFα mRNA expression following inhibition of the cyclooxygenase pathway in CBA mice

Total RNA was extracted from the spleens of indomethacin and ethanol treated CBA mice at day ten. RNA was used in a multiplex RT-SQ-PCR for IL-4, IFNγ and TNFα mRNA expression. No IL-4 mRNA was detected in any of the tissue samples analysed (data not shown). IFNγ mRNA production was significantly higher, over 21 fold, in CBA mice that received ethanol treated drinking water than in CBA mice receiving indomethacin treated water (Figure 5.9). TNFα mRNA was 1.4 fold higher in the spleens of CBA mice that received ethanol treated water than in the spleens of mice that were given indomethacin treated water (Figure 5.9).

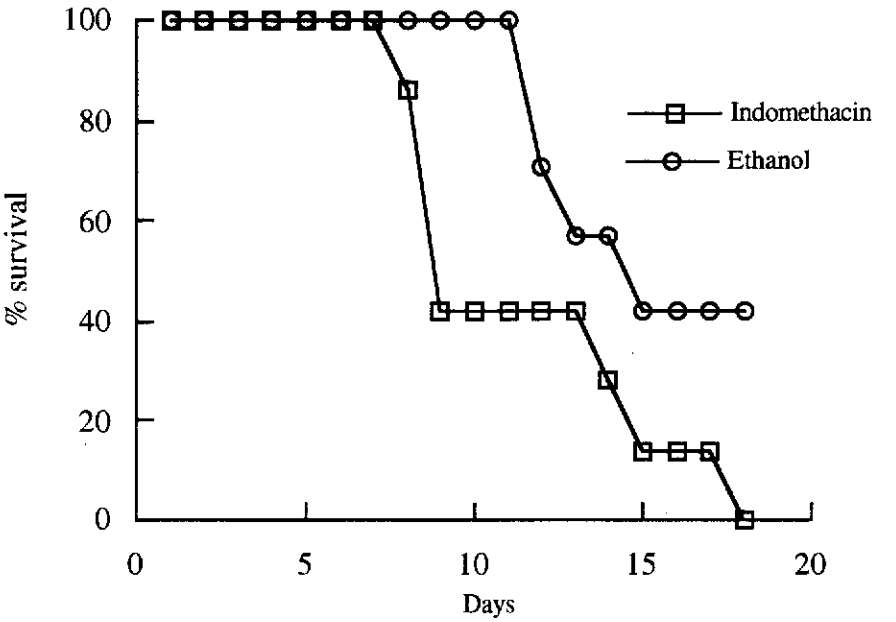

Figure 5.8 Mortality of CBA mice treated with indomethacin during chronic systemic candidiasis. CBA mice were challenged with $3x10^5$ viable *C. albicans* blastospores *iv*. Mice were provided with drinking water containing either 30μg/ml of indomethacin or 0.1% ethanol (n=7). Mortality was assessed over an eighteen day period.

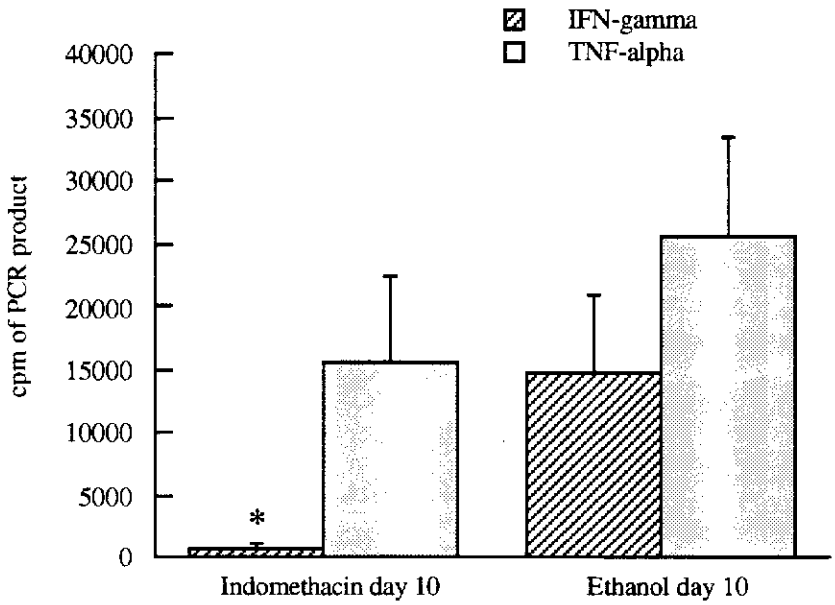

Figure 5.9 IFNγ and TNFα mRNA levels in the spleens of indomethacin treated and untreated mice CBA mice during chronic systemic candidiasis. CBA mice were challenged with $3x10^5$ viable *C. albicans* blastospores *iv*. Mice were provided with drinking water containing either 30μg/ml of indomethacin or 0.1% ethanol. On day ten three mice from each group were sacrificed and their spleens removed for RNA extraction. RNA was extracted and subjected to RT-SQ-PCR for IL-4, IFNγ and TNFα mRNA. No IL-4 mRNA was detected.
* IFNγ mRNA level significantly higher in mice receiving ethanol treated drinking water than in mice given indomethacin treated drinking water ($p<0.05$).

5.4 DISCUSSION

In 1986 Mosmann and colleagues described the presence of two subsets of T-helper (Th) cells derived from long term murine T-cell clones, which differed in the patterns of cytokines they secreted. Mosmann and colleagues categorised Th1 cells on the basis of IL-2, IFNγ and TNFβ secretion and Th2 cells on the basis of IL-4 and IL-5 secretion. The cytokine patterns produced correlated to cell mediated or DTH responses for Th1 and humoral and allergic responses for Th2. Since the initial classification the Th1 subset has been extensively linked to disease resistance in animal models (Romani *et al.*, 1991b; Scott and Kaufmann, 1991). Th2 responses, with the exception of parasitic diseases (Lange *et al.*, 1994) and autoimmune diseases (Scott *et al.*, 1994), are generally linked to disease exacerbation (Scott *et al.*, 1988; Scott and Kaufmann, 1991). IFNγ mRNA and IL-4 mRNA was assessed in BALB/c and CBA mice to test the hypothesis that BALB/c mice are more resistance to systemic candidiasis due to production of a Th1 response, or that CBA mice are more sensitive due to expression of a Th2 response.

BALB/c mice produced both IL-4 and IFNγ mRNA during the course of the infection with no significant dominance of one cytokine mRNA species over the other. However, IL-4 mRNA was consistently higher than IFNγ mRNA in the spleen of both strains of mice. Consequently there was no evidence of an overt Th1 response in the resistant BALB/c mice. In contrast, Romani *et al* (1993) reported a direct link between resistance to systemic candidiasis in BALB/c mice and production of a Th1 response. However, Romani *et al* (1994a) later suggested that resistance to systemic candidiasis may be more closely linked to the production of IL-12 rather than IFNγ. IL-12 mRNA was not measured in this study, as additional data indicated a Th1 response was not produced in acute systemic candidiasis by BALB/c mice.

In two independent experiments BALB/c mice were significantly protected from the acute effects of systemic candidiasis by treatment with neutralising Ab to IFNγ. Similar results were seen in CBA mice. In addition, the findings of this study are supported by data from other studies. Romani *et al* (1992b) failed to demonstrate increased sensitivity to primary systemic candidiasis in BALB/c mice following administration of neutralising Ab to IFNγ. While the effects of neutralising Ab studies may be due to Ab stabilisation of the IFNγ protein, findings of increased sensitivity in mice to systemic candidiasis following administration of exogenous IFNγ suggests this is not the case (Garner *et al.*, 1989). Finally, studies of Ab isotype production during systemic candidiasis failed to find evidence for a Th1 response in BALB/c mice (Finkelman *et al.*, 1990; Costantino, 1995b). Consequently, these studies suggest that BALB/c mice, in this model of systemic candidiasis, do not produce a Th1 response to primary infection.

Failure to detect a Th1 response in BALB/c mice during systemic candidiasis was in accordance with other disease models in which BALB/c mice are believed to have a genetic predisposition toward the production of a Th2 response rather than a Th1 response (Scott, 1991; Else *et al.*, 1994; Scott *et al.*, 1994; Hsieh *et al.*, 1995). The data obtained by Romani and colleagues (1993) may be explained in a comparative context. Romani and colleagues (1993) compared the T-helper responses of BALB/c mice to DBA mice. However, Hsieh *et al.* (1995) have shown that DBA mice have a greater genetic predisposition to a Th2 response than BALB/c mice. Therefore, the Th1 response noted in BALB/c mice by Romani and colleagues (1993) may be relative to the increased Th2 trend in the DBA mice.

Primary systemic candidiasis in CBA mice resulted in an early pattern of IL-4 and IFNγ mRNA expression similar to that of BALB/c mice. However, in contrast to BALB/c mice, the IL-4/IFNγ mRNA ratio increased in CBA mice in the later stages of the infection to, 6:1 day five and 11:1 on day seven. This "switch" to a Th2 like

response was coincident with maximal IL-2 mRNA production in these mice suggesting increased T-cell activation at this time. The change in the cytokine mRNA expression profile was also coincident with increased mortality in CBA mice as demonstrated in chapter 3 and in Figure 5.8. Hence, the increased IL-4 mRNA in relation to IFNγ mRNA may increase the sensitivity of CBA mice to chronic systemic candidiasis. Increased sensitivity may be due to the effects of Th2 cytokines on the effector mechanisms of macrophages, such as, NO production (Romani *et al.*, 1994b), which is down regulated by Th2 cytokines and up regulated by IFNγ (Sher *et al.*, 1992).

The timing of the shift in cytokine mRNA profiles in the CBA mice was consistent with shifts in cytokine patterns evident in other disease models and other models of systemic candidiasis (Romani *et al.*, 1991b; Romani *et al.*, 1994a; Wynn *et al.*, 1995). The timing of these responses indicates that Th2 or Th1 responses may be induced and possibly fixed from day three to seven of the infection. This indicates the initial differences in susceptibility between CBA and BALB/c mice were not due to T-cell effects, as differences were apparent within 24 hours of infection. However, it is possible the higher yeast load in CBA mice may drive a later change to a Th2 like response.

The findings of this study fit with a hypothesis suggested by Bretscher (1992), which seeks to explain the phenomenon of "low zone tolerance" in which administration of low doses of soluble Ag leads to cell mediated response while high Ag doses lead to Ab responses (Parish, 1972). In the Bretscher model, exposure to low Ag concentration leads to the initial generation of a cell mediated/Th1 response. The Th1 response can be "fixed" if exposure to low Ag concentration is extended. However, as Ag levels increase the immune response switches to an Ab/Th2 response. In the BALB/c mice this was seen with a low yeast load and transient repression of IL-4 mRNA compared to IFNγ mRNA and clearance of the infection. While this did not

result in an overt Th1 like response, immunisation with low Ag dose in BALB/c mice does lead to a Th1 pattern of cytokine mRNA production in secondary infection (chapter 7). In contrast, CBA mice had a higher Ag load and a similar initial decrease in IL-4 mRNA relative to IFNγ mRNA as seen in BALB/c mice, this may be a default to a neutral or more Th0 response. However, there was a later switch to a Th2 pattern of cytokine mRNA expression in the CBA mice, which correlated with increased mortality in these mice. As seen in BALB/c mice, there can be a shift in CBA mice to a predominantly IFNγ mRNA response to secondary infection if a sufficiently low inoculum of *C. albicans* is given (chapter 7).

The findings that neutralisation of IFNγ increased the resistance of mice to acute systemic candidiasis appears to be in conflict with the findings that in chronic infection reduction of IFNγ mRNA was coincident with increased mortality in CBA mice. However, IFNγ may have different roles in the innate and Ag specific responses to infection. In the early innate response, phagocyte activating cytokines such as TNFα and IL-1 are likely to be more important than IFNγ which should be produced maximally in a later Ag specific response. It has been demonstrated that inhibition of IFNγ increases the release of IL-1 from *L. major* infected BALB/c macrophages (Cillari *et al.*, 1989). Consequently, early IFNγ release, such as by NK cells, could inhibit monokine release and hence resistance to acute infection. In contrast, IFNγ may enhance resistance to chronic infection by preferentially generating a Th1 cell mediated response (Gajewski and Fitch, 1988), which has been shown to be protective in other models of systemic candidiasis (Romani *et al.*, 1991b; Romani *et al.*, 1992a; Romani *et al.*, 1993). Consequently, IFNγ appears to have a biphasic effect during systemic candidiasis. In the acute Ag non-specific stages of the immune response, IFNγ production was detrimental to disease resolution. In the chronic Ag specific stages of the immune response decreased IFNγ mRNA was associated with increased mortality.

Initial differences in susceptibility, if related to immune function, most likely reside within the innate response to infection. As cytokines such as TNFα, IL-1α and the colony stimulating factors (CSF), activate non-specific effector cells such neutrophils and macrophages, assessing the expression of these cytokines may provide information on disease susceptibility in CBA and BALB/c mice. To test this hypothesis, a range of cytokine mRNA species, that have been implicated in increasing the phagocytic killing of *C. albicans* (Djeu *et al.*, 1986; Karbassi *et al.*, 1987; Wang *et al.*, 1989b; Kullberg *et al.*, 1990; Tansho *et al.*, 1994) , or in protecting mice form systemic candidiasis (Van't Wout *et al.*, 1988; Kullberg *et al.*, 1990; Cenci *et al.*, 1991; Allendoerfer *et al.*, 1993), were measured during acute systemic candidiasis.

TNFα mRNA was substantially suppressed in BALB/c mice during the first seven days of infection compared to uninfected BALB/c mice. A similar suppression of TNFα mRNA was seen in the CBA mice at the beginning of the infection. However the level of TNFα mRNA increased in CBA mice during the infection, such that by day seven, infected and uninfected mice had similar TNFα mRNA levels. IL-1α mRNA was suppressed during infection in BALB/c mice. However IL-1α mRNA was only suppressed in CBA mice on day four of infection compared to uninfected CBA mice. Of the CSF's only M-CSF could be detected in either of the two strains of mice. Again this cytokine mRNA species was suppressed in the BALB/c mice during infection. In contrast M-CSF mRNA was elevated in the CBA mice during the infection.

The suppression of TNFα IL-1α and M-CSF mRNA species in BALB/c mice suggests these cytokines are unlikely to be involved in the differential resistance patterns seen between BALB/c and CBA mice. Additionally, while these cytokines have been implicated in increasing phagocytic killing (Djeu *et al.*, 1986; Karbassi *et al.*, 1987; Wang *et al.*, 1989b; Kullberg *et al.*, 1990; Tansho *et al.*, 1994) or in protecting mice from systemic candidiasis (Van't Wout *et al.*, 1988; Kullberg *et al.*,

1990; Cenci *et al.*, 1991; Steinsham and Waage, 1992; Allendoerfer *et al.*, 1993), the suppression of these cytokine mRNA species suggests these cytokines may have little effect in the spleens of these mice. Alternatively, mRNA levels may not be reflective of protein levels. However, at least *in vitro*, mRNA levels for most cytokines match expressed protein levels (Kelso, 1993). Alternatively, splenic cytokine production may be suppressed, maximal cytokine expression may be at the site of infection.

CBA mice consistently produced higher levels of mRNA for the cytokines measured than similarly infected BALB/c mice. The increased cytokine mRNA in CBA mice compared to BALB/c mice was likely a result of the higher yeast load in CBA mice. Hence, a dysfunction in the production of IL-2, IL-4, IFNγ, TNFα, IL-1α and M-CSF in CBA mice appears to be an unlikely explanation for the differences in the resistance phenotypes of CBA and BALB/c mice. It is possible that differences exist in the regulation of cytokines that were below that limit of detection such as, IL-3, GM-CSF and G-CSF. However, it seems likely that differences in these cytokines would have minimal effects given their low expression.

The elevated M-CSF mRNA, which follows the course of the infection in CBA mice, suggests that macrophages may be important in the resolution of infection in these mice. This is supported by evidence from other studies that indicate the importance of macrophages to resolution of systemic candidiasis (Baccarini *et al.*, 1986; Bistoni *et al.*, 1986; Karbassi *et al.*, 1987; Vecchiarelli *et al.*, 1988; Wang *et al.*, 1989b; Cenci *et al.*, 1991). Hence, macrophages may play a significant role in the resistance to systemic candidiasis. It is perhaps significant to note that while PMN cells are considered to be the major effector cell in systemic candidiasis, neither G-CSF, GM-CSF or IL-3 mRNA was detectable in any mice during primary systemic candidiasis. GM-CSF, G-CSF and IL-3 promote the growth, differentiation and activation of granulocytes and macrophages (Unanue, 1993).

It has been suggested that *C. albicans* induces a suppression of T-cell mediated immunity during infection (Marmor and Barnett, 1968; Canales *et al.*, 1969; Paterson *et al.*, 1971; Laforce *et al.*, 1975; Twomey, 1975; Fisher *et al.*, 1978; Podzorski *et al.*, 1990). This suppression of cell-mediated immunity may be due to excess PGE_2 production by macrophages (Witkin *et al.*, 1983; Witkin *et al.*, 1986) possibly as a result of the action of *C. albicans* mannan (Nelson *et al.*, 1991). Alternatively, it has been proposed that PGE_2 has its principle inhibitory action on the cell mediated immune response by selectively inhibiting Th1 responses (Betz and Fox, 1991; Snijdewint *et al.*, 1993; van der Pouw Kraan *et al.*, 1995) and promoting Th2 responses (Roper *et al.*, 1990; Roper *et al.*, 1995; van der Pouw Kraan *et al.*, 1995). A consistent finding of this study was a generalised reduction in the production of many of the cytokine mRNA species measured during infection compared to the level of mRNA found in uninfected mice. It is possible the reduction in cytokine mRNA in infected mice was due to increased PGE_2 production during infection. Additionally, it was possible the "Th2 like" switch, noted in the spleens of CBA mice during chronic systemic candidiasis was due to the action of PGE_2. To test these two hypotheses PGE_2 production was inhibited by administration of indomethacin. Indomethacin is an inhibitor of the cyclooxygenase pathway, which produces PGE_2 from the metabolism of arachadonic acid.

Indomethacin treatment did not increase the resistance of CBA mice to acute systemic candidiasis. Contrary to expected results, inhibition of the cyclooxygenase pathway consistently increased the sensitivity of BALB/c mice to acute systemic candidiasis and increased the sensitivity of CBA mice to chronic infection. Hence, PGE_2 does not appear to be a mediator of increased sensitivity in CBA mice and may in fact be protective in both strains of mice. Analysis of the cytokine response in CBA mice treated with indomethacin during systemic candidiasis showed that inhibition of the cyclooxygenase pathway decreased the IFNγ mRNA production in these mice. The decreased IFNγ mRNA in CBA mice correlated with increased mortality. Therefore

contrary to expected results inhibition of the cyclooxygenase pathway resulted in inhibition of IFNγ mRNA. There was no evident effect on TNFα mRNA production. These data are in conflict with findings of *in vitro* experiments were PGE_2 selectively inhibits IFNγ production (Betz and Fox, 1991; Snijdewint *et al.*, 1993). Incomplete inhibition of PGE_2 production does not appear to be the reason for these results as BALB/c mice had substantial GIT stasis as a result of treatment, presumably due to inhibition of prostaglandin production and therefore smooth muscle contraction. Therefore inhibition of the cyclooxygenase pathway can have opposing effects to those seen *in vitro*, when given *in vivo*.

Inhibition of the cyclooxygenase pathway did not result in increased cytokine production during systemic candidiasis, indicating this was not the cause of the generalised reduction in cytokine mRNA production seen during infection. Reduced cytokine mRNA production may be a result of the reduced total splenic T-cell population in both CBA and BALB/c mice during systemic candidiasis as demonstrated by reduced Thy1.2 staining (Costantino, 1995a), a phenomenon that has been reported by others (Kullberg *et al.*, 1993). Maximal suppression of $Thy1.2^+$ cell numbers was seen in the spleen at day three in CBA and BALB/c mice. Therefore, minimal splenic T-cell numbers correlated to the time of maximal splenic cytokine mRNA expression in infected mice. Consequently, the cytokine mRNA levels per cell may be increased in the spleen during infection. Alternatively, it is possible that cells are primed in the spleen but have suppressed transcription until they reach the site of inflammation in the periphery. This may serve to prevent immune mediated damage at inappropriate sites. The high level of cytokine mRNA production in the control mice may represent a basal production of cytokine mRNA, without concomitant translation. Whatever the mechanism for the suppression in cytokine mRNA in infected compared to uninfected mice, this suppression was consistently seen in the spleen in all experiments performed over a three year period.

In conclusion, there was no evidence of a Th1 response in BALB/c mice during primary systemic candidiasis in this mouse model. Additionally, there was no evidence of differential expression of the IL-4 and IFNγ mRNA between CBA and BALB/c mice in acute systemic candidiasis. However, there was a later shift to a Th2 like response in CBA mice that was coincident with increased mortality in these mice. As the initial cytokine mRNA responses were similar in both strains of mice this later response in the CBA mice may be a sequelae to the inability to clear the infection and the higher resultant yeast load. The difference in susceptibility between CBA and BALB/c does not reside in the ability to produce macrophage and neutrophil activating cytokines mRNA species, as CBA mice consistently produce more mRNA for these cytokines than BALB/c mice. The increased sensitivity of CBA mice was not reversed by inhibition PGE_2 production. In fact, inhibition of PGE_2, increased the sensitivity of CBA and BALB/c mice to systemic candidiasis and reduced the expression of IFNγ mRNA in treated mice. Finally, IFNγ appears to have opposing roles in acute and chronic infection, that may be due to differential effects on the innate and Ag specific immune response.

CHAPTER SIX

CYTOKINE mRNA PROFILES IN NON-LYMPHOID TISSUE

Abstract

Systemic candidiasis manifests as a multiorgan disease in which the level of *C. albicans* colonisation varies depending on tissue type. Histologically the disease is manifested mostly in the kidney and the brain of CBA and BALB/c mice. Cytokine profiles and T-helper phenotypes can vary from tissue to tissue. Consequently, cytokine mRNA profiles were assessed in various organs to determine if the pattern of tissue colonisation by *C. albicans* could be explained by cytokine mRNA expression. Cytokine mRNA levels in the kidneys of infected mice were found to be similar to, or lower, than those of uninfected mice. This suggests that high colonisation levels in the kidneys during systemic candidiasis are due to insufficient activation of the predominantly neutrophil infiltrate. In contrast, the brain had a lower yeast load and resolved the infection more rapidly than the kidney. In the brain cytokine mRNA levels were high during infection and corresponded to a Th1 phenotype with IFNγ mRNA predominating over IL-4 mRNA. Hence, macrophages and/or microglia found in the abscesses within the brain may be substantially more activated than cells found within the kidney. The liver and the lungs had no histological evidence of infection which was consistent with a lack of cytokine mRNA expression in these tissues. In summary, this study has found that cytokine patterns vary from tissue to tissue during systemic candidiasis. These differences may explain the inability of mice to adequately control yeast proliferation in the kidneys. The differential pattern of cytokine mRNA expression in murine tissues has been demonstrated in two inbred strains of mice. Finally, there was no difference in the expression of cytokine mRNA species between CBA and BALB/c mice that can be used to explain the differences in susceptibility between these two strains of mice to systemic candidiasis.

6.1 INTRODUCTION

Cytokine production has typically been monitored *in vitro* and *in vivo* in splenic cells or peripheral blood cells in disease as a measure of the ensuing immune response. However, evidence suggests that cytokine production and T-helper phenotype may vary from tissue to tissue within the same animal in response to the same stimulation (Troutt and Lee, 1989) or pathogen (Scott and Kaufmann, 1991). Hence, cytokine production in the spleen and the peripheral blood may not be a reflection of the cytokine responses at other sites of infection. During systemic candidiasis there is considerable heterogeneity in the colonisation levels of *C. albicans* from tissue to tissue (Chapter 3; Hector *et al.*, 1982; Ashman and Papadimitriou, 1987; Ashman and Papadimitriou, 1988). The differences in tissue distribution may be due to; differential ability of tissues to adequately clear the infection, resistance to initial colonisation or differential targeting of *C. albicans* to tissues. The ability of tissues to clear established *C. albicans* colonisation or resist colonisation may be reflected by the cytokine patterns and/or level of cytokine mRNA produced at the site of infection. Alternatively, if certain tissues are not targeted by *C. albicans* it might be supposed that these tissues have minimal cytokine production.

The two most heavily infected organs in systemic candidiasis are the kidney and the brain (chapter 3). Of these two organs, the kidney consistently demonstrated the higher level of colonisation (chapter 3). Histological and immunohistological analysis of infected kidneys revealed an immune infiltrate composed predominantly of neutrophils. In contrast, the inflammatory infiltrate of brain was composed of macrophages and/or microglia cells with only minimal numbers of neutrophils. Both organs had only a mild T-cell infiltration during infection with few or no $CD3^+$ T-cell infiltration of abscesses (chapter 3). The liver of infected mice had a high yeast load, as assessed by quantitative yeast culture, but consistently failed to show histological evidence of infection (chapter 3). Similarly, the lungs had little infection as determined

by histology (chapter 3). Finally, the heart had the lowest yeast load with no histological evidence of infection.

The cytokine mRNA profiles of various non-lymphoid tissues were examined to test the hypothesis that cytokine expression varies between tissues and that differences in cytokine mRNA profiles correlate with the ability of tissues to either prevent or clear infection. Cytokine mRNA levels were assessed in the; brain, kidney, liver, lungs and heart. As the brain and the kidney were the only two of these organs to become significantly infected, these organs were targeted for a larger range of cytokines than the other less involved tissues. The liver, lungs and heart were monitored for a more discrete range of cytokine mRNA species to determine if cytokine production prevented infection or if cytokine production reflected a lack of inflammatory response.

IFNγ and IL-4 mRNA species were measured as indicators of Th1 and Th2 responses respectively (Mosmann *et al.*, 1986). IL-2 mRNA was measured as an indicator of T-cell activation. Other cytokines such as TNFα, IL-1α, M-CSF, GM-CSF and G-CSF were measured as these cytokines either increase the phagocytic killing of *C. albicans in vitro* (Djeu *et al.*, 1986; Karbassi *et al.*, 1987; Wang *et al.*, 1989b; Kullberg *et al.*, 1990; Tansho *et al.*, 1994), or protect mice from systemic candidiasis when used *in vivo* (Van't Wout *et al.*, 1988; Kullberg *et al.*, 1990; Cenci *et al.*, 1991; Steinsham and Waage, 1992; Allendoerfer *et al.*, 1993).

6.2 MATERIALS AND METHODS

Tissues used in this chapter were taken from the mice used in chapter 3 and 5. Tissues were collected from CBA and BALB/c mice following *iv* inoculum of $1x10^6$ viable *C. albicans* (2.5.2) Tissues were removed and immediately frozen in liquid N_2 and stored at -80°C until needed. Total RNA was extracted (2.8.7) from tissues one to four days post infection and used for single or multiplex RT-SQ-PCR (2.9) and conventional RT-PCR (2.8.6) to assess cytokine mRNA production.

Statistical comparisons between two groups were performed by the Mann-Whitney U test. For multiple comparisons, statistical significance was assessed by the Kruskal-Wallis one way Anova. Post-Hoc comparisons were performed with the Mann-Whitney U test with an appropriate downsizing of the α value to compensate for multiple analyses.

6.3 RESULTS

6.3.1 *In vivo* cytokine mRNA expression in the brain during systemic candidiasis

6.3.1.1 IL-2, IL-3, IL-4, IFNγ, and TNFα mRNA production

The production of IL-2, IL-3, IL-4, IFNγ and TNFα mRNA was measured in the brain of infected (day four) and uninfected CBA and BALB/c by multiplex RT-SQ-PCR. IL-2 and IL-3 mRNA were below the level of detection in the brain of infected and uninfected BALB/c and CBA mice (data not shown). IL-4, IFNγ and TNFα mRNA species were not detectable in the brain of uninfected BALB/c and CBA mice by multiplex RT-SQ-PCR (Figure 6.1a and 6.1b).

IFNγ mRNA expression was significantly higher than IL-4 mRNA expression in the brain of BALB/c mice during systemic candidiasis (Figure 6.1a). Additionally, TNFα mRNA was significantly higher than IFNγ mRNA in the brain of infected BALB/c. In the brain of infected BALB/c mice TNFα mRNA expression was 2 fold higher than IFNγ mRNA expression and 7 fold higher than IL-4 mRNA expression. IFNγ mRNA expression was 3 fold higher than IL-4 mRNA expression (Figure 6.1a).

There was a similar pattern of cytokine mRNA expression in the brain of CBA mice to that seen in the brain of infected BALB/c mice (Figure 6.1a and 6.1b). IFNγ mRNA levels were significantly higher than IL-4 mRNA levels. Levels of TNFα mRNA in the brain of infected CBA mice were significantly higher than IFNγ mRNA. TNFα mRNA levels, in the brain of infected CBA mice, were 3 fold higher than IFNγ mRNA and over 15 fold higher than IL-4 mRNA. IFNγ mRNA was over 5 fold higher than IL-4 mRNA (Figure 6.1b). Additionally, IL-4, IFNγ and TNFα mRNA levels were all significantly higher in the brain of infected CBA mice

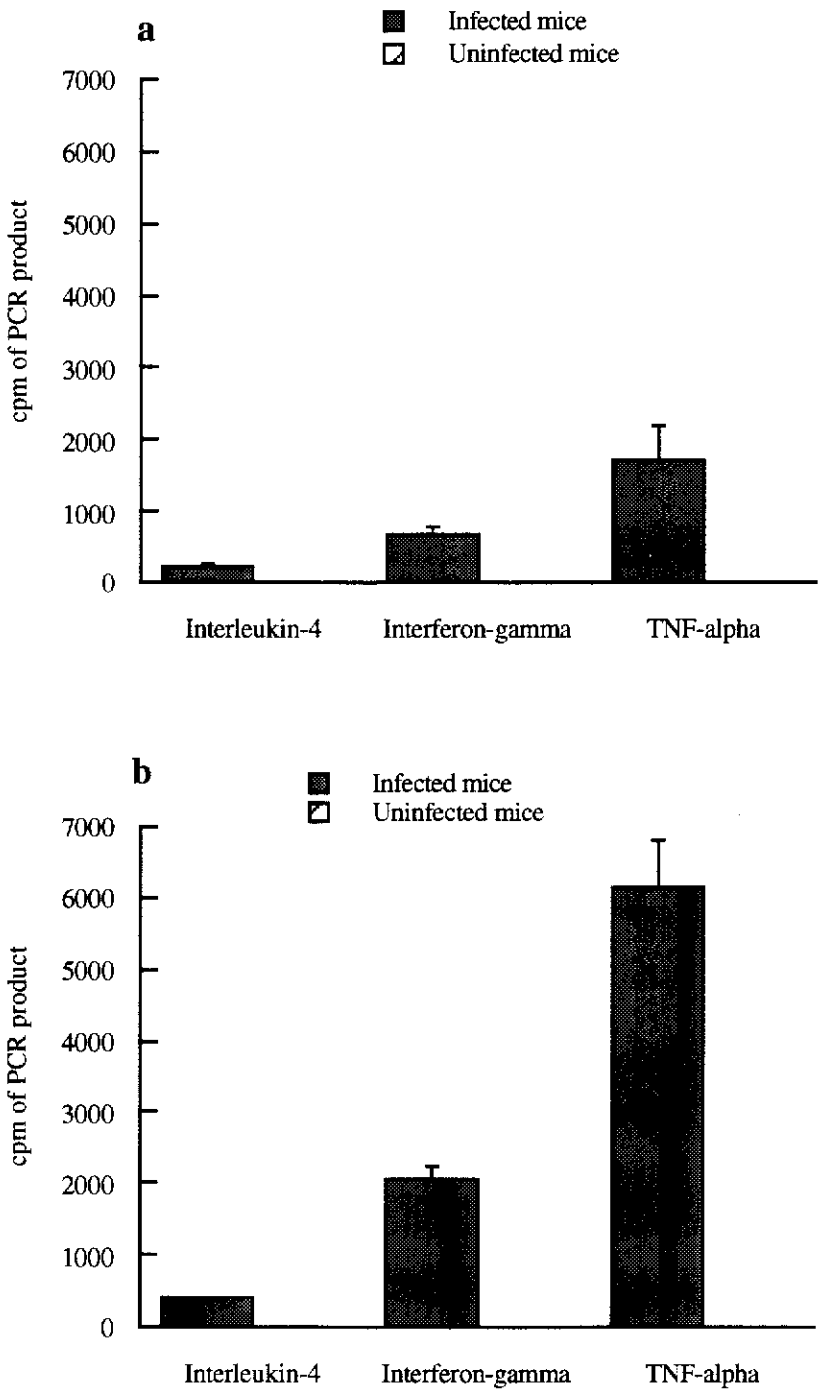

Figure 6.1 Multiplex RT-SQ-PCR for IL-2, IL-3, IL-4, IFNγ and TNFα mRNA in the brain of BALB/c mice (a) and CBA mice (b). IL-2 and IL-3 mRNA were below the level of detection (not shown). IL-4, IFNγ and TNFα mRNA were below the level of detection in uninfected mice (n=2/strain). Values shown are the mean ± SEM of infected BALB/c and CBA mice (n=4/stain) on day four of infection following inoculation of $1x10^6$ viable *C. albicans* blastospores.

than in the brain of infected BALB/c mice by, 1.7, 3.1 and 3.6 fold, respectively (Figure 6.1a and b).

6.3.1.2 Interleukin-1α, GM-CSF and G-CSF mRNA production

The cDNA produced for 6.3.1.1 was used in a multiplex RT-SQ-PCR for the detection of IL-1α and GM-CSF mRNA in the brain of infected and uninfected BALB/c and CBA mice. Both BALB/c and CBA mice produced IL-1α mRNA in response to infection (Figure 6.2). Levels of IL-1α mRNA was significantly higher in the brain of infected CBA mice (1.8 fold) than in brain of infected BALB/c mice. While IL-1α mRNA was detected in the brain of uninfected BALB/c and CBA mice this was lower than in infected mice (3.7 and 6.1 fold respectively).

GM-CSF was not detectable in the brain of infected or uninfected CBA or BALB/c mice using multiplex RT-SQ-PCR. Furthermore, GM-CSF mRNA could not be detected in the brain of infected or uninfected mice by the more sensitive conventional 40 cycle RT-PCR (data not shown). Conventional 35 cycle RT-PCR was employed for the qualitative detection of G-CSF mRNA in the brain of infected BALB/c and CBA mice. G-CSF mRNA was not detectable in the brain of infected mice using conventional RT-PCR (data not shown).

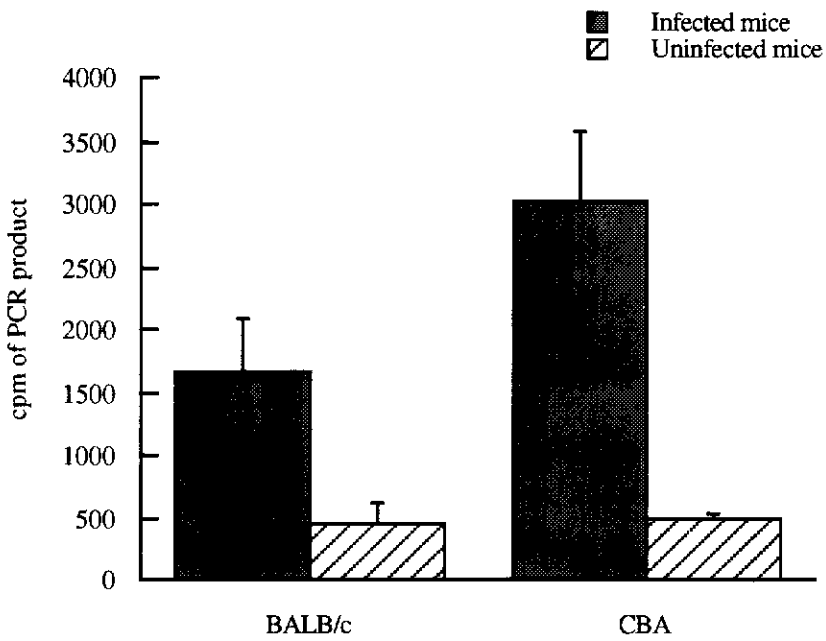

Figure 6.2 RT-SQ-PCR for IL-1α mRNA in the brain of BALB/c and CBA mice. Values shown are the mean ± SEM of uninfected BALB/c and CBA mice (n=2/strain) and infected BALB/c and CBA mice (n=4/stain) on day four of infection with $1x10^6$ viable *C. albicans* blastospores.

6.3.1.3 M-CSF mRNA production

The cDNA produced for 6.3.1.1 was used in a RT-SQ-PCR for the detection of M-CSF mRNA in the brain of infected and uninfected CBA and BALB/c mice. Both BALB/c and CBA mice produced M-CSF mRNA in response to infection (Figure 6.3). M-CSF mRNA expression was 1.9 fold higher in infected CBA mice than in infected BALB/c mice. However, the difference in M-CSF expression between CBA and BALB/c mice was not significant. M-CSF mRNA was produced in the brain of uninfected BALB/c and CBA mice (Figure 6.3) but was over 2 and 3 fold lower than in infected BALB/c and CBA mice, respectively.

6.3.2 *In vivo* cytokine mRNA expression in the kidneys during systemic candidiasis

6.3.2.1 Interleukin-4 and IFNγ mRNA production.

Multiplex RT-SQ-PCR was performed on total RNA extracted from the kidneys of infected and uninfected CBA and BALB/c mice with the primers for IL-4 and IFNγ. Results described are from four infected mice per strain plus four uninfected mice per strain. Data shown in Figure 6.4 is from day three post infection. In both BALB/c and CBA mice the level of IL-4 and IFNγ mRNA was at the limit of detection with multiplex RT-SQ-PCR. There was no significant difference in the level of IL-4 mRNA production between infected and uninfected BALB/c mice. Additionally, there was no significant difference in the level of IFNγ mRNA production between infected and uninfected BALB/c mice. Similar data was obtained in CBA mice, in that, no significant difference was evident between the level of expression of either IL-4 or IFNγ mRNA in infected compared to uninfected CBA mice (Figure 6.4). Comparable results were obtained in the kidney of CBA and BALB/c mice at day four of systemic candidiasis (data not shown).

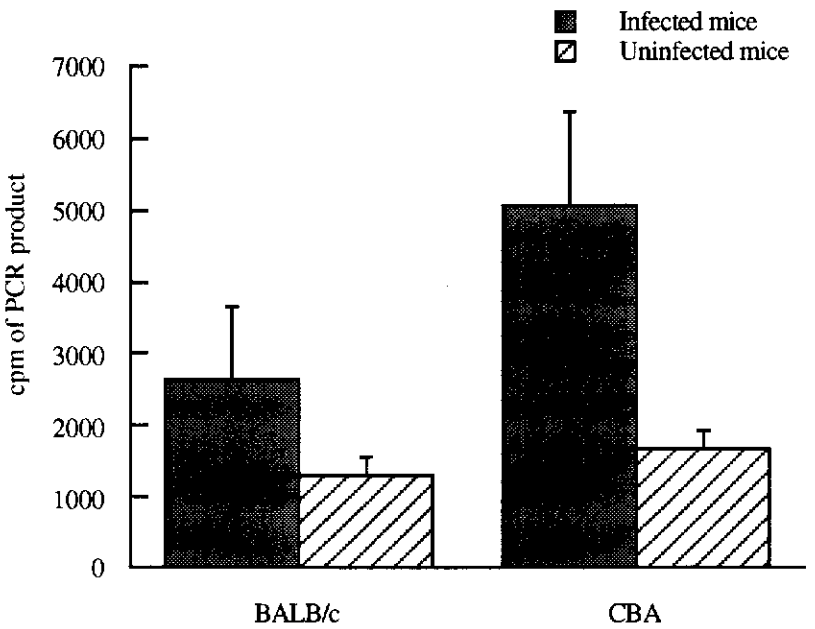

Figure 6.3 RT-SQ-PCR for M-CSF mRNA in the brain of BALB/c and CBA mice. Values shown are the mean ± SEM of uninfected BALB/c and CBA mice (n=2/strain) and infected BALB/c and CBA mice (n=4/stain) on day four of infection with $1x10^6$ viable *C. albicans* blastospores.

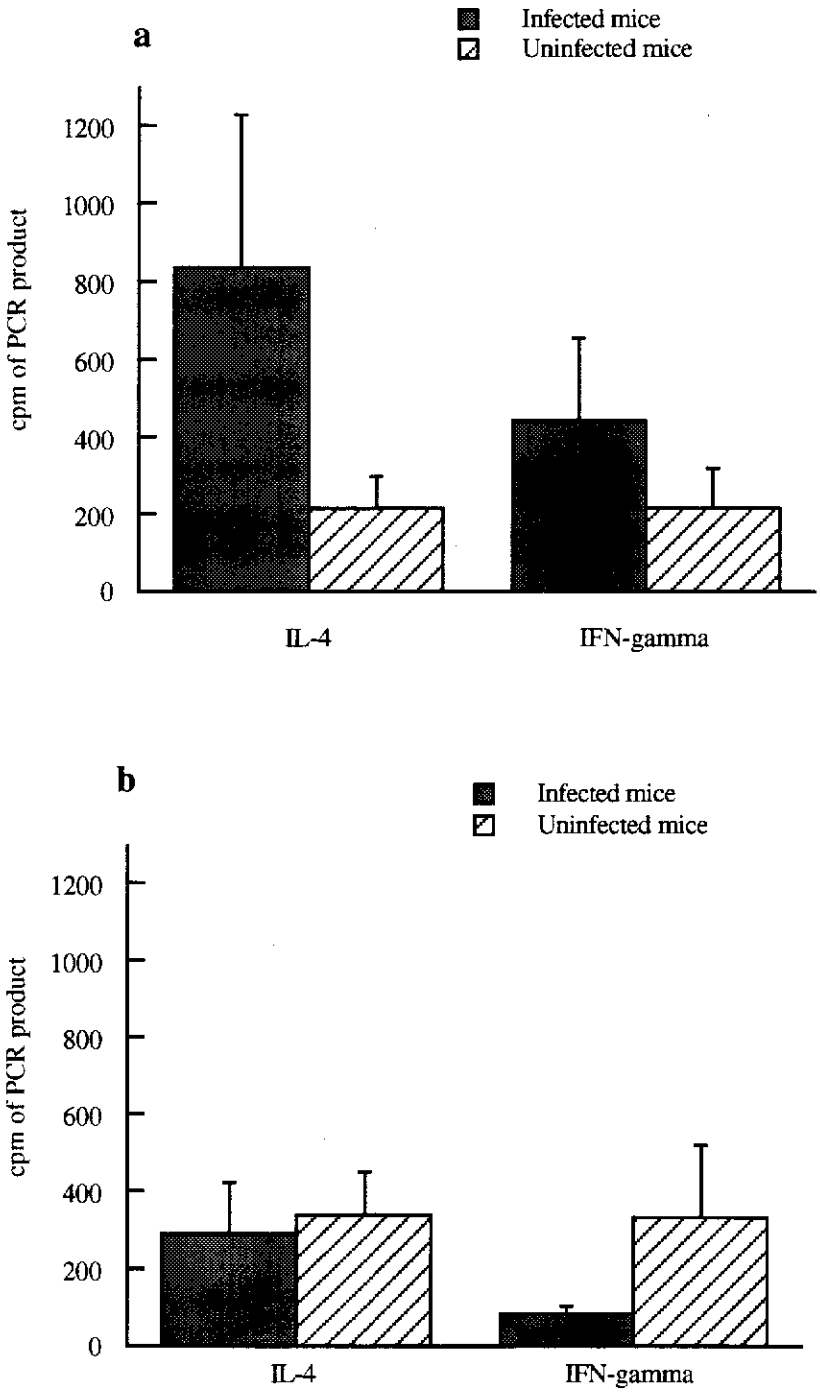

Figure 6.4 Multiplex RT-SQ-PCR for IL-4 and IFNγ mRNA in the kidney of BALB/c mice (a) and CBA mice (b). Values shown are the mean ± SEM of uninfected (n=4/strain) and infected BALB/c and CBA mice (n=4/stain) on day three of infection with $1x10^6$ viable *C. albicans* blastospores.

The low level of IL-4 and IFNγ mRNA expression in kidney tissue was consistently demonstrated in up to three independent experiments with varying inoculum sizes of *C. albicans*. In CBA mice, IFNγ mRNA was expressed at higher levels from day one to two than at day four. However, in all experiments IL-4 mRNA levels were consistently higher than that of IFNγ mRNA. IL-4 mRNA levels increased while IFNγ mRNA levels decreased in the kidneys of infected CBA mice, such that in some experiments only IL-4 mRNA was detectable at day four (data not shown). In infected BALB/c mice, IFNγ mRNA was also produced earlier in the kidney. However, in general, IL-4 and IFNγ mRNA species were lower in the kidneys of BALB/c mice than in the kidneys of infected CBA mice (data not shown).

6.3.2.2 IL-2, IL-3 and TNFα mRNA production.

Levels of IL-2, IL-3 and TNFα mRNA production in the kidneys of BALB/c and CBA mice was assessed by multiplex RT-SQ-PCR. There was no significant difference in the level of TNFα mRNA expression between infected and uninfected BALB/c mice (Figure 6.5). In CBA mice there was a 9 fold increase in the production of TNFα mRNA in infected CBA mice compared to uninfected mice (Figure 6.5). However, the difference in TNFα mRNA production was not significant as TNFα mRNA levels in the kidney were low and variable. The lack of a significant difference between the levels of cytokine production in the kidneys of infected and uninfected mice was consistent for all cytokine mRNA species. This was likely a result of the low level of cytokine mRNA expression in the kidney, which was at the limit of detection, and hence accuracy, (4.3.6) in BALB/c and CBA mice.

IL-2 and IL-3 mRNA was not detected in the kidneys of infected or uninfected CBA and BALB/c mice by multiplex RT-SQ-PCR. In addition, IL-3 mRNA was not detected in infected mice when assayed by conventional 40 cycle RT-PCR (data not shown).

6.3.2.3 IL-1α, GM-CSF and G-CSF mRNA production.

Multiplex RT-SQ-PCR was used to assess IL-1α and GM-CSF mRNA production in the kidney of infected mice. IL-1α mRNA was not detected by RT-SQ-PCR in the kidneys of infected or uninfected BALB/c or CBA mice. However, there was readily detectable levels of IL-1α mRNA using the more sensitive, conventional 40 cycle RT-PCR in the kidneys of infected BALB/c and CBA mice (Data not shown). In contrast GM-CSF mRNA was not detectable with RT-SQ-PCR or conventional RT-PCR (Data not shown).

The expression of G-CSF mRNA was assessed by conventional 35 cycle RT-PCR. G-CSF mRNA was not detectable by conventional RT-PCR in the kidney of infected BALB/c or CBA mice (data not shown).

6.3.2.4 M-CSF mRNA production

M-CSF mRNA production was assessed in the kidneys of infected and uninfected mice by RT-SQ-PCR. M-CSF mRNA levels were below the limit of detection with the RT-SQ-PCR in the kidneys of both strains of mice, whether infected or uninfected (data not shown). However, M-CSF mRNA was detected by conventional 40 cycle RT-PCR in the kidney of BALB/c and CBA mice during systemic candidiasis (data not shown).

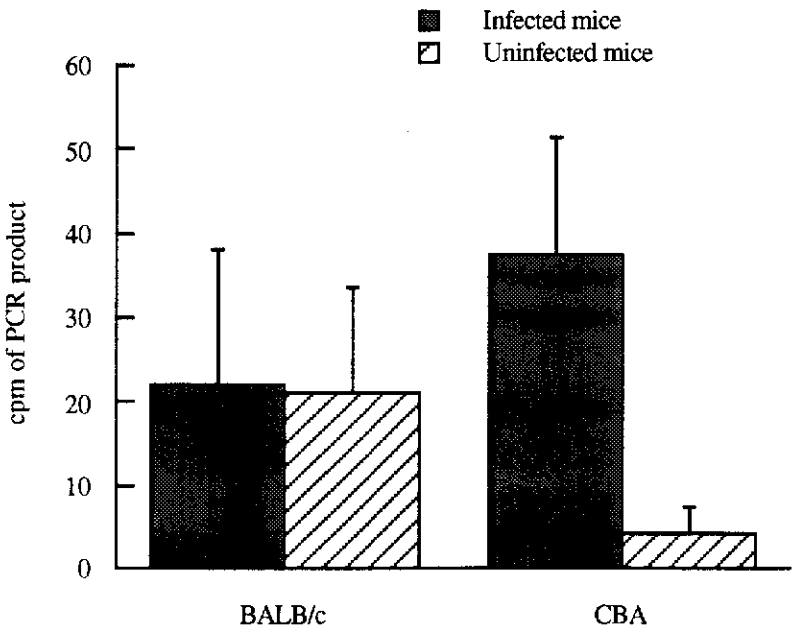

Figure 6.5 Multiplex RT-SQ-PCR for TNFα, IL-2 and IL-3 mRNA in the kidneys of BALB/c mice and CBA mice. IL-2 and IL-3 mRNA was below the limit of detection with multiplex RT-SQ-PCR (not shown). Values shown are the mean ± SEM of TNFα mRNA levels in infected (n=4/stain) and uninfected (n=4/strain) BALB/c and CBA mice on day three of infection with $1x10^6$ viable *C. albicans* blastospores.

6.3.3 *In vivo* cytokine mRNA expression in the liver during systemic candidiasis

Histological examination of the liver of infected BALB/c and CBA mice failed to find evidence of *C. albicans* colonisation or abscess formation (chapter 3). Consequently analysis of cytokine production was limited, with only IL-4, IFNγ, IL-2, IL-3 and TNFα measured by RT-SQ-PCR on day four of infection. Relatively high levels of IL-4 mRNA was detected in the liver of both CBA and BALB/c mice (Figure 6.6a and b). However, there was no significant difference in the level of IL-4 mRNA between infected and uninfected mice. Additionally, there was no significant difference in the level of IL-4 mRNA production between infected BALB/c and infected CBA mice (Figure 6.6a and b). Furthermore, there was no significant difference in the level of IFNγ and TNFα mRNA produced by infected compared to uninfected mice. Finally, there was no significant difference in the level of IFNγ and TNFα mRNA in the liver of infected CBA mice compared to infected BALB/c mice.

The levels of IL-4, IFNγ and TNFα mRNA were compared in the liver of each individual strain of mouse during infection by Kruskal-Wallis one way Anova. Only in CBA mice was there a significant difference in the production of these cytokine mRNA species. Post-Hoc analysis revealed that there was significantly more IL-4 mRNA than TNFα mRNA in the liver of infected CBA mice.

M-CSF and IL-1α mRNA species were demonstrated in the liver of infected BALB/c and CBA mice by conventional RT-PCR. However, IL-3, GM-CSF and G-CSF mRNA species were not detectable in the liver of infected BALB/c and CBA mice by conventional RT-PCR (data not shown).

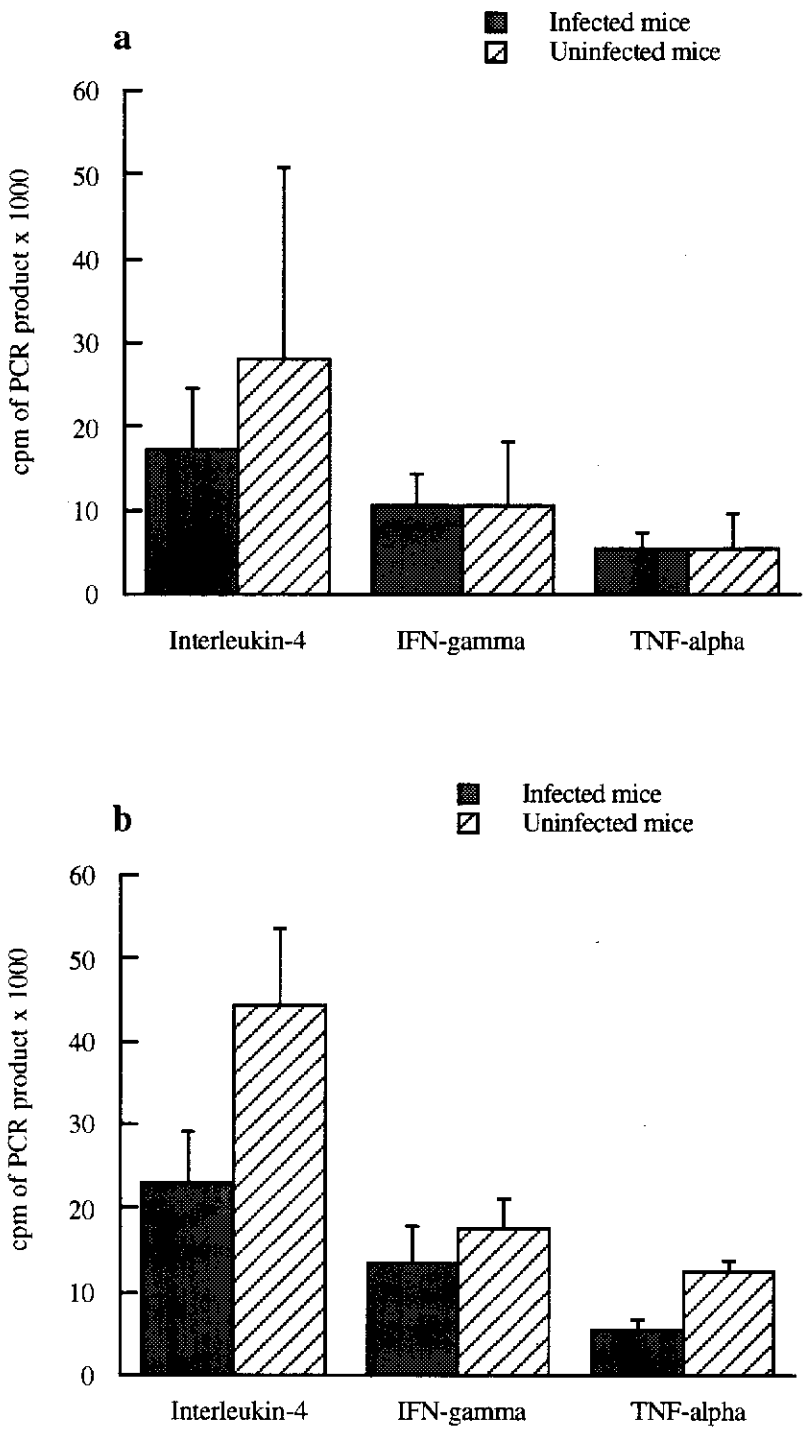

Figure 6.6 Multiplex RT-SQ-PCR for IL-2, IL-3, IL-4, IFNγ and TNFα mRNA in the liver of BALB/c mice (a) and CBA mice (b). IL-2 and IL-3 mRNA was below the level of detection by RT-SQ-PCR (not shown). Values shown are the mean ± SEM of uninfected BALB/c and CBA mice (n=2/strain) and infected BALB/c and CBA mice (n=4/stain) on day four of infection with $1x10^6$ viable *C. albicans* blastospores.

6.3.4 *In vivo* cytokine mRNA expression in the lungs during systemic candidiasis

As with the liver, there was no overt yeast infection in the lungs of infected mice. Consequently, a limited range of cytokine mRNA species were measured on day four of infection to determine if cytokine production protected from systemic candidiasis or if lack of cytokine gene expression suggested the lungs were not sites of *C. albicans* multiplication.

Multiplex RT-SQ-PCR for IL-4, IFNγ and TNFα mRNA in the lungs of infected and uninfected mice revealed a pattern of cytokine gene expression similar to that seen in the liver. However, there was in general, lower cytokine mRNA expression in the lungs than was seen in the liver (data not shown). There was no significant difference between the levels of IL-4, IFNγ or TNFα mRNA in the lungs of infected BALB/c mice compared to uninfected BALB/c (Figure 6.7a). Similar results were seen in the level of IL-4, IFNγ or TNFα mRNA species in the lungs of CBA mice. In addition there was no significant difference between the level of IL-4, IFNγ and TNFα mRNA species between infected CBA compared to infected BALB/c mice.

Although there was a trend to higher IL-4 mRNA than IFNγ mRNA, which was in turn, higher than TNFα mRNA in the lungs of infected BALB/c mice (Figure 6.7a), these differences were not significant by the Kruskal-Wallis one way Anova testing. A similar trend was seen in infected CBA mice (Figure 6.7b). However, in contrast to BALB/c mice there was a significant difference in the expression of IL-4, IFNγ and TNFα mRNA in the lungs of infected CBA mice. Post-Hoc testing revealed that IL-4 mRNA levels were significantly higher than TNFα mRNA.

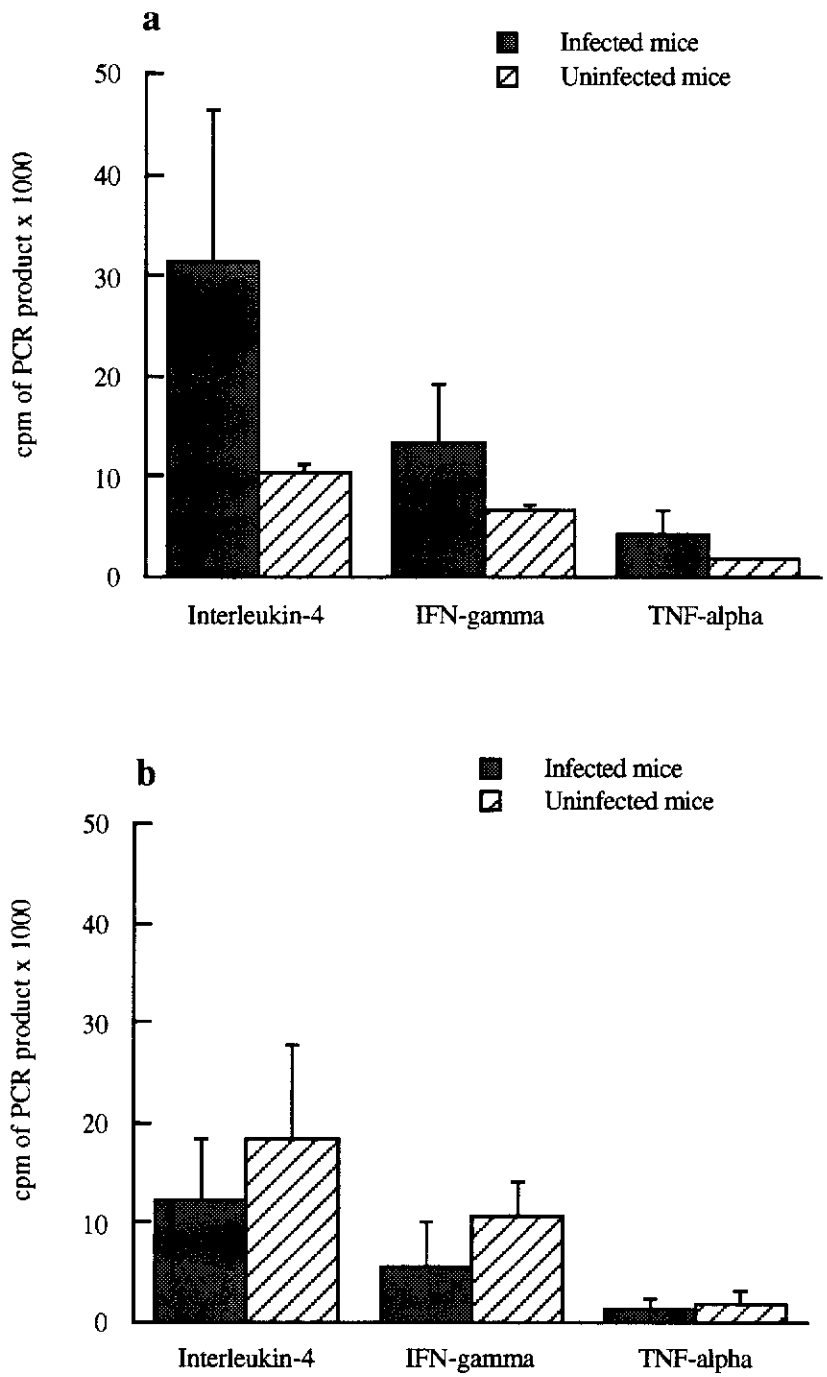

Figure 6.7 Multiplex RT-SQ-PCR for IL-2, IL-3, IL-4, IFNγ and TNFα mRNA in the lungs of BALB/c mice (a) and CBA mice (b). IL-2 and IL-3 was not detectable in the lungs of CBA and BALB/c mice by RT-SQ-PCR (not shown). Values shown are the mean ± SEM of uninfected BALB/c and CBA mice (n=2/strain) and infected BALB/c and CBA mice (n=4/stain) on day four of infection with $1x10^6$ viable *C. albicans* blastospores.

IL-1α mRNA was demonstrated in the lungs of infected BALB/c and CBA mice by conventional RT-PCR. However, M-CSF, G-CSF, GM-CSF and IL-3 mRNA was not detectable in the lungs of infected BALB/c and CBA mice by conventional RT-PCR (data not shown).

6.3.5 *In vivo* cytokine mRNA expression in the heart during systemic candidiasis

The heart is often the site of infection in humans (Luna and Tortoledo, 1993) and in other animal models of systemic candidiasis (Hurley and Fauci, 1975). However, in this study, the heart as with the liver and lungs, showed no evidence of tissue pathology as assessed by histology (chapter 3). The heart also had the lowest level of colonisation by *C. albicans* (chapter 3). For this reason a restricted set of cytokine RT-SQ-PCR was carried out with infected heart tissue. Cytokine mRNA production was assessed on day four of primary systemic candidiasis and compared to uninfected mice.

Total RNA, extracted from the heart of infected and uninfected BALB/c and CBA mice, was subjected to multiplex RT-SQ-PCR using IL-4, IFNγ, TNFα, IL-2 and IL-3 primers (Figure 6.8). IL-4 mRNA expression was low in the heart of infected CBA mice and was below the level of detection in infected BALB/c mice (Figure 6.8a). IL-4 mRNA was below the limit of detection in the heart of uninfected BALB/c and CBA mice (Figure 6.8a and 6.8b). IFNγ mRNA was readily detectable in the heart of in infected, but not in uninfected, BALB/c mice (Figure 6.8a). In contrast, similar levels of IFNγ mRNA were detected in the heart of infected and uninfected CBA mice (Figure 6.8b). There was a significant reduction in TNFα mRNA levels, by over 5 fold, in the heart of infected CBA mice compared to uninfected CBA mice. However, the level of TNFα mRNA production in the heart of infected BALB/c mice was similar to that found in the heart of uninfected BALB/c mice (Figure 6.8a)

Kruskal-Wallis one way Anova testing revealed that there was no significant difference between the levels of IL-4, IFNγ and TNFα mRNA species in the heart of infected CBA mice (Figure 6.8b). In contrast, there was a significant difference in the levels of IL-4, IFNγ and TNFα mRNA in the heart of infected BALB/c mice. Post-Hoc analysis revealed that TNFα mRNA was higher than IFNγ mRNA in the heart of infected BALB/c mice and that IFNγ mRNA was higher than IL-4 mRNA. In addition, infected BALB/c mice produced more IFNγ and TNFα mRNA in the heart than was evident in the heart of infected CBA mice (Figure 6.8a and 6.8b)

IL-2 and IL-3 mRNA species were below the limit of detection with the multiplex RT-SQ-PCR in the heart of infected and uninfected CBA and BALB/c mice (data not shown). However, IL-3 mRNA was detectable in the heart of infected BALB/c and CBA mice by conventional RT-PCR, IL-2 mRNA expression was not assessed by this method. Conventional RT-PCR demonstrated considerable levels of IL-1α and M–CSF mRNA but not G-CSF and GM-CSF mRNA in the heart of infected BALB/c and CBA mice (data not shown).

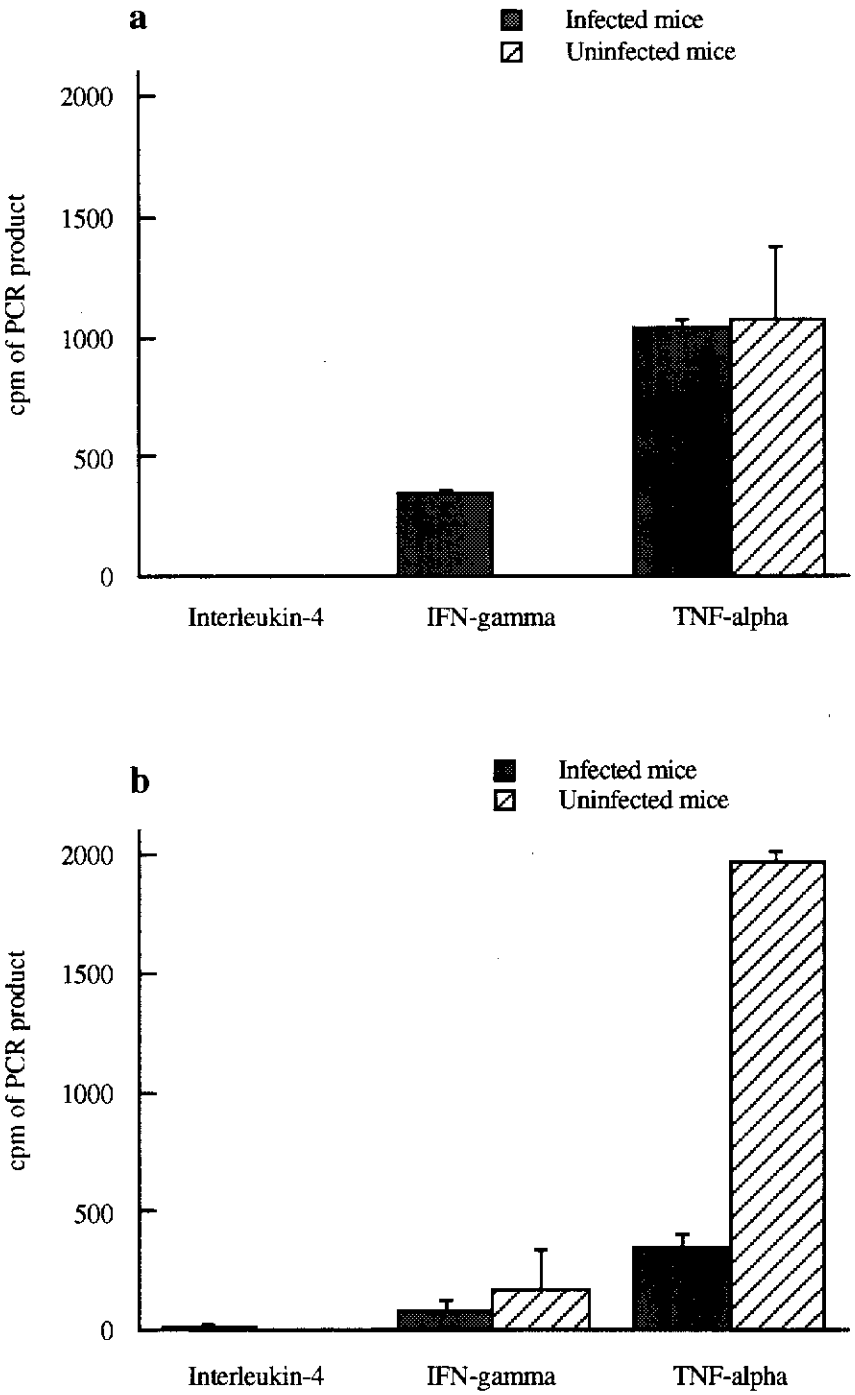

Figure 6.8 Multiplex RT-SQ-PCR for IL-2, IL-3, IL-4, IFNγ and TNFα mRNA in the heart of BALB/c mice (a) and CBA mice (b). IL-2 and IL-3 mRNA was below the limit of detection with multiplex RT-SQ-PCR in both BALB/c and CBA mice (not shown). Values shown are the mean ± SEM of uninfected BALB/c and CBA mice (n=2/strain) and infected BALB/c and CBA mice (n=4/stain) on day four of infection with $1x10^6$ viable *C. albicans* blastospores.

6.4 DISCUSSION

The immune response to invading pathogens is frequently measured *in vitro* or *in vivo* in lymphoid organs or peripheral blood. However, patterns of cytokine expression and T-helper phenotype can vary from tissue to tissue (Troutt and Lee, 1989; Scott and Kaufmann, 1991). Hence, cytokine production in lymphoid organs or in peripheral blood may not be reflective of those at the site of inflammation in non-lymphoid organs. During systemic candidiasis there is considerable heterogeneity in the colonisation levels and clearance rates of *C. albicans* from lymphoid and non-lymphoid tissues (Chapter 3; Hector *et al.*, 1982; Ashman and Papadimitriou, 1987; Ashman and Papadimitriou, 1988). Consequently, a range of cytokine mRNA species was assessed in multiple organs of mice with systemic candidiasis, to test the hypothesis that *C. albicans* tissue distribution patterns are reflected by cytokine mRNA expression patterns. This study has demonstrated differential patterns and levels of cytokine gene expression in non-lymphoid organs. High cytokine mRNA expression in the brain of infected mice was concomitant with resolution of infection at this site. In contrast, there was minimal cytokine expression in the kidney of infected mice which was concomitant with failure to substantially clear the yeast burden in this tissue.

In the brain of infected BALB/c and CBA mice there was significantly higher levels of IFNγ mRNA than IL-4 mRNA. The increased IFNγ mRNA compared to IL-4 was consistent with a Th1 response to infection (Mosmann *et al.*, 1986). Other cytokine mRNA species increased in the brain of infected mice were IL-1α, TNFα, and M-CSF. All of the cytokine species that were raised in the brain of infected mice, with the possible exception of IL-4, which was considerably lower than IFNγ, have been shown to increase the candidacidal activity of phagocytic cells or protect mice from systemic candidiais. IFNγ and TNFα increase the activation of macrophages and PMN and increase their candidacidal activity (Djeu *et al.*, 1986; Wang *et al.*, 1989b;

Marodi and Johnston, 1993; Redmond *et al.*, 1993; Marodi *et al.*, 1994; Tansho *et al.*, 1994; Blasi *et al.*, 1995). IL-1α is protective in models of intra cerebral candidiasis (Mazzolla *et al.*, 1991). M-CSF stimulates increased candidacidal activity of monocytes (Wang *et al.*, 1989b) and protects mice from systemic candidiasis when given *iv* (Cenci *et al.*, 1991). TNFα is produced by murine macrophages in response to *C. albicans* and protects mice from infection when administered systemically (Allendoerfer *et al.*, 1993). Finally, early increases in the production of TNFα, IL-6 and IL-1β mRNA transcripts in the brain of mice has been associated with increased resistance to intra cerebral inoculation of *C. albicans* (Blasi *et al.*, 1994).

The high level of cytokine mRNA expression indicates that inflammatory cells in the brain, which are predominantly mononuclear, F4/80 and or Mac-1 positive, may be highly activated from locally produced cytokines. This is consistent with the increased MHC class II staining found in the brain of mice with systemic candidiasis (chapter 3). Hence, cytokine production and immigrant cell populations in the brain of mice with systemic candidiasis indicates the expression of a highly efficient immune response to *C. albicans*. Additionally, the cytokine mRNA expression pattern correlated to the induction of a Th1 phenotype, a phenotype that has been extensively linked to disease resistance in numerous disease models (Heinzel *et al.*, 1989; Salgame *et al.*, 1991; Yamamura *et al.*, 1991; Clerici and Shearer, 1993), including systemic candidiais (Romani *et al.*, 1991b; Romani *et al.*, 1993).

It is possible the Th1 cytokines or high levels of cytokines such as TNFα in CBA mice increased their sensitivity to infection. Susceptibility to cerebral malaria in mice has been associated with Th1 responses or high TNFα responses (Grau *et al.*, 1989; de Kossodo and Grau, 1993). Additionally, TNFα overproduction has been linked to tissue pathology in other central nervous system (CNS) diseases (Freund *et al.*, 1992). However, the role of TNFα and Th1 responses in diseases of the CNS is contradictory, as high TNFα and IFNγ production can also be associated with survival

(Gazzinelli *et al.*, 1993; Wesselingh *et al.*, 1994). Additionally, the similar pattern of cytokine gene expression in the brain of both strains of mice suggests the higher level of cytokine mRNA expression in CBA mice was related to yeast load and not a causative factor in the increased resistance seen in these mice. This was supported by the findings that, in most tissues, CBA mice expressed higher levels of cytokine mRNA species than BALB/c mice. Nevertheless, it is likely that TNFα production in the brain of both CBA and BALB/c mice contributes to the symptoms associated with systemic candidiasis such as weight loss (Tracey *et al.*, 1990).

A possible consequence of cytokine production in the brain is damage to the integrity of blood-brain barrier and hence enhanced migration of both yeast and inflammatory cells into the CNS. It is well established that cytokines such as TNFα and IL-1β increase the permeability of the blood-brain barrier (Anthony *et al.*, 1997; Saija *et al.*, 1995). In fact IL-1β at high concentrations in cell culture can cause separation of the CNS endothelial cell junctions (Wang *et al.*, 1993). It is possible that cytokines in the blood are responsible for increased blood-brain barrier permeability (Saija *et al.*, 1995) which contributes, at least in part, to the initial entry of *C. albicans* into the CNS. CNS produced cytokines may exacerbates this effect. Hence, the increased yeast load in the brain of CBA mice maybe a result of increased systemic and CNS derived cytokines in these mice compared to BALB/c mice. However, as detailed above, it is more likely that high yeast load in the brain of CBA mice is a function of an overall increase in yeast load. However, in both strains of mice it is likely that CNS produced cytokines, as well as those transported from the blood to the brain (Gutierrez *et al.*, 1993), contribute to increased permeability of the blood-brain barrier and enhance migration of inflammatory cells and perhaps yeast into the CNS.

The production of a Th1 response in the brain of CBA and BALB/c mice appears to be contradicted by the lack of IL-2 mRNA, a T-cell derived cytokine, and the absence of T-cells within abscesses of the brain (chapter 3). Similar findings have been reported

by others, in that IL-2 mRNA product and T-cells surface markers, were low or undetectable by quantitative PCR in the brain of mice with systemic candidiasis (Ashman *et al.*, 1995). Hence, it is likely that most of the cytokine mRNA detected in the brain of CBA and BALB/c mice during systemic candidiasis was due to cells other than T-cells, such as natural killer (NK) cells, mast cells, microglia and neuronal cells (Ibrahim *et al.*, 1979; Troutt and Lee, 1989; Ben-Sasson *et al.*, 1990; Lechan *et al.*, 1990; Tyor *et al.*, 1992; Yao *et al.*, 1992; Sebire *et al.*, 1993). Consequently, the Th1 response in the brain may be best categorised as a Th1 like or a type I response.

In contrast to the brain, the level of cytokine mRNA expression in the kidneys of infected mice was consistently at the limit of detection with the RT-SQ-PCR. In no instance was cytokine mRNA production significantly higher in the infected mice than in uninfected mice. Additionally, there was no evident expression of the genes for G-CSF, GM-CSF, IL-2 or IL-3 in the kidney of infected or uninfected mice.

The low cytokine gene expression and predominantly neutrophil infiltrate in the kidney (chapter 3) was associated with high colonisation levels and persistence of *C. albicans* in this organ. This was particularly evident in CBA mice. Hence, activation of neutrophils at the inflammatory site by locally produced cytokines may be minimal. This would result in decreased neutrophil killing of *C. albicans* as optimal neutrophil action is contingent on the presence of macrophage and T-cell derived cytokines (Djeu *et al.*, 1986; Djeu and Blanchard, 1987; Ashman and Papadimitriou, 1990; Djeu *et al.*, 1990; Sweeney *et al.*, 1994). While neutrophils themselves are capable of producing IL-1, TNF and IL-6 in response to *C. albicans* (Palma *et al.*, 1992), it is likely the lack of cytokines such as IL-4, IFNγ and IL-2 result in limited amplification of the immune response and reduced neutrophil cytokine expression. The limited capacity of neutrophils to control infection in the kidneys of mice has been illustrated in other studies. In mice protected from systemic candidiasis by treatment with IL-4 antagonists, increased resistance to disease was associated with a replacement of the

typical PMN infiltrate with a lymphomononuclear infiltrate (Romani *et al.*, 1992c; Puccetti *et al.*, 1994). Further, resistance to secondary systemic candidiasis was due to the presence of highly candidacidal macrophages and not neutrophils in the spleens of infected mice (Bistoni *et al.*, 1988; Vecchiarelli *et al.*, 1988).

It is interesting to note that, although neutrophils are believed to be the first line of defence in resistance to systemic candidiasis, G-CSF, GM-CSF and IL-3 mRNA were not detected in any tissue during systemic candidiasis. The only CSF mRNA detected in the kidney was for M-CSF, a cytokine that is specific for macrophages and has no action on neutrophils (Unanue, 1993). BALB/c mice had considerable infiltration of activated macrophages in the kidneys during primary systemic candidiasis (chapter 3). Hence, the presence of M-CSF in the kidneys of BALB/c mice may increase the candidacidal activity of immigrant macrophages (Karbassi *et al.*, 1987) and increase resistance to systemic candidiasis (Cenci *et al.*, 1991). This may in part, explain the increased resistance of BALB/c mice compared to CBA mice, as control of infection in the most heavily affected organ would decrease dissemination to other less affected organs. Additionally, control of the infection in the kidney would decrease the chances of renal failure in infected animals.

In contrast to the brain and kidney, the liver and lungs showed no evidence of infection as assessed by histology (chapter 3). The lack of inflammatory response in the liver and lungs was coupled with a lack of significant cytokine gene translation. A surprising finding of this study was that TNFα mRNA levels were consistently lower than IL-4 and IFNγ in the liver and lungs of infected mice. In contrast, in organs with histological evidence of infection such as the kidney and the brain, TNFα mRNA levels were consistently higher than other cytokine mRNA species. This suggests that inflammatory responses in the liver and lungs were minimal and that these organs control infection without significant activation of the cytokine cascade. This is likely to be due to the large resident phagocytic cell populations in these organs which

prevent establishment of infection and allow these organs to clear substantial numbers of *C. albicans* from the peripheral blood (Schwocho and Moon, 1981; Sawyer, 1990; Sawyer *et al.*, 1990)

During systemic candidiasis, the heart of infected mice had the lowest yeast load of all the tissues assayed in these studies. Histological examination failed to find evidence of yeast infection, though in other models, myocardial infection does occur (O'Black *et al.*, 1980). In the heart of infected BALB/c mice, cytokine mRNA expression was consistent with a Th1 response to infection with IFNγ mRNA levels higher than IL-4 mRNA levels. In contrast there was no significant difference between IFNγ mRNA levels and IL-4 mRNA levels in the heart of infected CBA mice. Additionally, TNFα mRNA levels in the heart of infected CBA mice was significant lower than in the heart of uninfected CBA and infected BALB/c mice. The lack of histological evidence and low level of *C. albicans* recovery from infected heart tissues makes it likely that cytokine response seen in the heart were due to the presence of peripheral blood cells, rather than to an inflammatory lesion. If this is the case, then the pattern of responses in the peripheral blood are similar to that of the brain in the BALB/c mice but not in CBA mice.

The lower level of TNFα mRNA and IFNγ mRNA in the heart of CBA compared to the heart of BALB/c is contrary to the pattern observed in other tissues in that typically, the CBA mice produced similar to, or higher levels of cytokine mRNA, than BALB/c mice. This difference, if due to peripheral blood cell rather than infection in the heart tissue, may allow the yeast to better establish infection in CBA mice compared to BALB/c mice. BALB/c mice may have a greater candidacidal response in the peripheral blood than CBA mice which would reduce the spread of the *C. albicans* from tissue to tissue. Additionally, cells immigrating from the blood to infected organs would be more activated in BALB/c mice than in CBA mice. Alternatively, the cytokine pattern seen in the heart may be a response to a small localised infection such

as occurs in human infection (Luna and Tortoledo, 1993). This may not be present in CBA mice or may be more effectively responded to in the BALB/c mice.

A caveat to the conclusions discussed in this Thesis is that the presence of cytokine producing cells in the vascular compartment of tissues could mask the cytokine mRNA profile produced by cells in the parenchyma of tissues. It would be expected that the vascular compartment would contribute similar cytokine mRNA profiles to each tissue, although, depending on the vascularity of the tissue, the level of cytokine mRNA contributed by the vascular compartment may differ. However, the finding that the cytokine mRNA profiles differed from tissue to tissue suggests that the mRNA from cells in the vascular compartment does not mask, nor contribute significantly to the tissue specific cytokine mRNA pattern. However, in tissues with low cytokine mRNA profiles the presence of cytokine mRNA from the vascular compartment could have a more marked effect than in other tissues. This does not appear to be the case in these studies as the organs with the lowest cytokine mRNA expression, the heart, lungs and kidneys, all had different cytokine mRNA profiles. In the brain of uninfected mice there was little or no cytokine mRNA expression. This suggests minimal expression of cytokine mRNA in the peripheral blood in this and other organs in uninfected mice. However there was readily detectable levels of cytokine mRNA in other tissues of uninfected mice. The low cytokine mRNA expression in the brain of uninfected mice is likely due to the immunoprivileged status of this organ. These data suggest the major source of cytokines in the tissues of infected and uninfected mice was from cells within the parenchyma of the tissue rather than from within the vascular compartment of these tissues. A possible exception to this was the heart, which had a large volume of peripheral blood compared to tissue.

In conclusion, this study has demonstrated that cytokine gene expression patterns vary from tissue to tissue in mice with systemic candidiasis. This has been shown independently in two inbred strains of mice and demonstrates that cytokine production

in the spleen during infection in animal models may not reflect immune responses occurring in the non-lymphoid tissues. The cytokine production patterns in the brain of both healer and non-healer mice indicate a highly activated immune response to *C. albicans*, consistent with the production of a Th1 or type I response. This supports the data obtained in chapter 5, in that, CBA mice do not have increased sensitivity, at least in the acute infection with *C. albicans*, because of an inappropriate Th2 response. There was minimal cytokine gene expression in the kidney during the infection. Minimal cytokine gene expression was coupled with a predominantly neutrophil infiltrate with few T-cells and macrophages. These factors were associated with reduced ability to clear the infection in the kidneys compared to the brain. Hence, the kidney is highly susceptible to infection by *C. albicans*, possibly due to minimal activation of immigrant neutrophils. This was particularly evident in CBA mice, macrophage infiltration of the kidneys was more pronounced in BALB/c mice.

CHAPTER SEVEN

SECONDARY SYSTEMIC CANDIDIASIS

Abstract

Mice immunised with a sublethal *iv* inoculum of *C. albicans* have increased resistance to systemic candidiasis compared to naive mice. The mRNA expression patterns in secondary and primary systemic murine candidiasis were compared. This was performed to determine if different cytokine mRNA expression patterns could explain the increased resistance of mice to secondary infection with *C. albicans*. In particular, IFNγ and IL-4 mRNA levels were assayed to determine if the increased resistance seen in mice with secondary infection was due to the induction of a Th1 response. Several tissues, the spleen, kidney and brain of infected CBA and BALB/c mice were analysed to determine if the tissue specific patterns of cytokine production, demonstrated in primary infection (chapter 6), also occur in secondary infection.

The consistent findings of experiments detailed in this chapter were that IFNγ mRNA expression dominated that of IL-4 mRNA in the tissues of BALB/c mice during secondary infection. IL-4 but not IFNγ mRNA was measured in the spleen and kidneys of BALB/c mice during primary systemic candidiasis. Consequently during secondary systemic candidiasis Th1 like responses dominated in the kidney, whereas Th2 like responses dominated during primary systemic candidiasis. In the spleen, the cytokine profiles correlated to a Th0 like response during secondary systemic candidiasis and a Th2 like response during primary systemic candidiasis. Parallel results were seen in the kidneys and spleens of CBA mice. Additionally, there was evidence that cytokine responses in the spleens of BALB/c and CBA mice were inhibited or minimised during primary and secondary systemic candidiasis compared to non-immunised mice. This supports data obtained in previous experiments (chapter 5) and suggests that cytokine responses are dampened in this organ possibly as a mechanism of localising the immune response and hence any potential bystander effects to the sites of infection. In conclusion, these results support the hypothesis that increased resistance of immunised mice to systemic candidiasis was due to cytokine gene expression of a predominantly Th1 pattern. These data also support previous conclusions that CBA mice do not have a relative deficiency in the expression of cytokine mRNA compared to BALB/c mice.

7.1 INTRODUCTION

Various experimental models have studied the effects of systemic candidiasis in naive (Hector *et al.*, 1982; Bibas Bonet de Jorrat *et al.*, 1989), and immune mice (Hector *et al.*, 1982; Ashman and Papadimitriou, 1988). These models have been employed to determine the components of the immune response necessary for resistance to primary and secondary infection. Most studies have found that resistance to systemic candidiasis is enhanced by immunisation (Hector *et al.*, 1982; Ashman and Papadimitriou, 1988), suggesting a role for adaptive immune responses. Hector *et al* (1982) demonstrated significant protection from secondary systemic candidiasis in six inbred strains of mice following immunisation. Resistance to primary and secondary infection in these mice did not appear to be due to Ab production. Another study, with the yeast strain used in this Thesis, also failed to demonstrate a link between resistance to primary systemic candidiasis and Ab production (Costantino, 1995b). These findings, plus the fact that passive transfer of Ab protects only DTH positive mice (Kagaya *et al.*, 1981), suggests that increased resistance to systemic candidiasis in immunised mice is not due to protective Ab.

The lack of demonstrable Ab mediated protection suggests that protective adaptive immune responses are T-cell mediated. Various studies have demonstrated that T-cell mediated effects, such DTH and T-cell proliferate responses, are associated with resistance to systemic candidiasis (Kagaya and Fukazawa, 1981; Ashman, 1990). Indeed BALB/c mice, as used in this study, have a greater DTH response to *C. albicans* than CBA mice (Hector *et al.*, 1982; Ashman, 1990). However, Hector *et al* (1982) found that while resistance to systemic candidiasis correlated to DTH in some inbred strain of mice this was not always the case. Hence, other mechanisms are involved in resistance to systemic candidiasis.

The DTH response correlates to expression of a Th1 response to infection (Mosmann *et al.*, 1986). The Th1 response has been associated with resistance to both primary and secondary systemic candidiasis (Romani *et al.*, 1991b; Romani *et al.*, 1993). Alternatively, the Th2 response has been associated with increased sensitivity to infection, suggesting that Ab has minimal role in resistance to systemic candidiasis (Romani *et al.*, 1991b). Additionally, T-cells have been directly implicated in the resolution of systemic candidiasis (Romani *et al.*, 1992b; Sieck *et al.*, 1993). Previous studies have demonstrated that BALB/c and CBA mice can be protected from systemic candidiasis by immunisation with a sublethal inoculation of *C. albicans* (chapter 3). Consequently cytokine mRNA expression was assessed in mice with secondary systemic candidiasis to determine if protection afforded by immunisation correlated to the expression of Th1 responses. Additionally, as previous data have shown that cytokine patterns vary from tissue to tissue during primary systemic candidiasis (chapter 6) the expression of cytokine mRNA was monitored in the spleen, kidneys and brain of mice to determine if this also occurs during secondary systemic candidiasis.

7.2 MATERIALS AND METHODS

Tissues used in this chapter were taken from the mice used in chapter 3 (3.3.2). Tissues were collected from immune or naive CBA and BALB/c mice following an *iv* inoculum of $1x10^6$ viable *C. albicans* (2.5.2). Tissues were removed and immediately frozen in liquid N_2 and stored at -80°C until needed. Total RNA was extracted as described (2.8.7) and used in a cDNA reaction (2.9.1). The cDNA samples from each tissue were subject to single or multiplex PCR for cytokine mRNA measurement according to the described method (2.9). Samples were also subjected to conventional PCR (2.8.6)

7.3 RESULTS

7.3.1 Secondary systemic candidiasis in BALB/c mice

Immunisation of BALB/c mice with a sublethal dose of *C. albicans* led to protection from subsequent re-challenge (chapter 3). Studies were undertaken to determine the cytokine mRNA profile of immunised BALB/c mice during systemic candidiasis. Mice were immunised *iv* with $1x10^5$ viable *C. albicans* and re-challenged *iv* with $1x10^6$ viable *C. albicans* 25 days later. Tissues were removed, frozen in liquid nitrogen and stored at -80°C until needed. Total RNA were extracted and cytokine mRNA profiles were determined by RT-SQ-PCR.

The cytokine mRNA response to secondary infection were compared to that of uninfected mice and naive mice given a primary infection. Naive mice received non pyrogenic saline at the time of immunisation and were inoculated with $1x10^6$ viable *C. albicans* along with previously immunised mice 25 days later. Uninfected mice received non pyogenic saline at both time points. The cytokine mRNA profile in the spleen, kidney and brain was assessed at day one and four of secondary systemic candidiasis. Cytokine mRNA expression in uninfected mice and in mice with primary systemic candidiasis was assessed in the spleen, kidneys and brain on day four only.

7.3.1.1 *In vivo* cytokine mRNA expression in the spleen

Spleens were removed from infected and uninfected mice. Total RNA was extracted and used in a multiplex RT-SQ-PCR for the detection of TNFα, IL-2, IL-4 and IFNγ mRNA. Conventional PCR was exploited to assess G-CSF and GM-CSF mRNA production.

SQ-RT-PCR assessment of TNFα, IL-2, IL-4 and IFNγ mRNA production.

During secondary infection, immunised mice produced IFNγ, IL-4 and TNFα mRNA at days one and four. TNFα mRNA levels were slightly higher than IFNγ and IL-4 mRNA levels (Figure 7.1). Additionally, IFNγ mRNA was slightly higher than IL-4 mRNA in immunised mice on day one and four. There was a slight reduction in the level of all cytokine mRNA species measured on day four compared to day one in the spleens of BALB/c mice with secondary systemic candidiasis. Naive mice undergoing a primary infection did not express detectable levels of either IL-4 or IFNγ mRNA in the spleen (Figure 7.1). However, mice with primary systemic candidiasis did produce TNFα mRNA. The IFNγ and IL-4 mRNA expression patterns were confirmed in a repeat experiment (data not shown). IL-2 mRNA was not detected in any group of infected or uninfected mice (data not shown)

Uninfected mice produced the highest levels of TNFα and IL-4 mRNA, with similar levels of IFNγ mRNA to those seen in immune mice at days one and four (Figure 7.1). However, of importance is the finding that of the infected BALB/c mice, IFNγ mRNA was detectable only in mice with secondary systemic candidiasis. Furthermore, IFNγ mRNA was higher than IL-4 in the spleen of mice with secondary systemic candidiasis, while in uninfected mice, IFNγ was lower than IL-4. These results are similar to those seen in chapter 5, in which IL-4 mRNA levels were consistently higher than IFNγ mRNA levels in the spleens of uninfected mice and mice with primary systemic candidiasis (Figure 7.1).

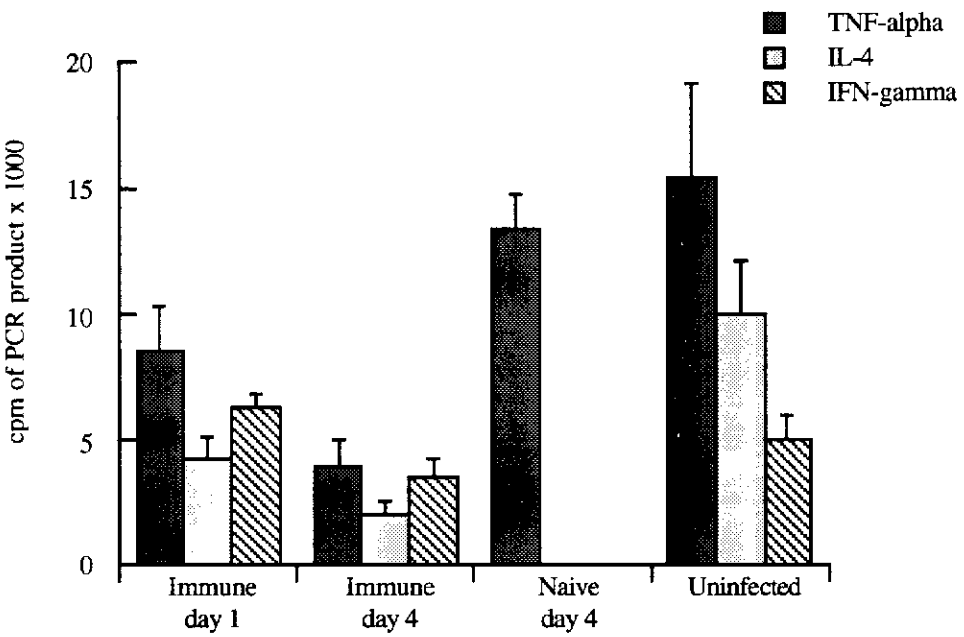

Figure 7.1 TNFα, IL-4 and IFNγ mRNA production in the spleen of immunised and naive BALB/c mice as assessed by multiplex SQ-RT-PCR. Immune BALB/c mice were given $1x10^5$ viable *C. albicans* blastospores *iv* and rechallenged 25 days later with $1x10^6$ viable *C. albicans* blastospores. Naive mice were given saline in the first inoculation followed by $1x10^6$ viable *C. albicans* blastospores at the time of secondary inoculation. Uninfected mice received only sterile non-pyrogenic saline. Spleens were removed at the times indicated, after the second inoculation, and the cytokine mRNA profiles were assessed by multiplex RT-SQ-PCR. Values shown are mean of 5-6 mice per group ± SEM.

Qualitative assessment of G-CSF and GM-CSF mRNA production

Conventional PCR was used for a qualitative assessment of G-CSF and GM-CSF mRNA expression in the spleen of BALB/c mice. G-CSF mRNA was not detected in the spleen during secondary or primary systemic candidiasis or in uninfected mice. In contrast, GM-CSF was detected on day one of secondary candidiasis, but could not be detected at day four nor could it be detected in uninfected mice or mice with primary systemic candidiasis (data not shown).

7.3.1.2 *In vivo* cytokine mRNA expression in the kidneys

Kidneys were removed from infected and control mice. Total RNA was extracted and used in a multiplex RT-SQ-PCR for the detection of TNFα, IL-2, IL-4 and IFNγ mRNA. Conventional PCR was exploited to assess G-CSF and GM-CSF mRNA production.

SQ-RT-PCR assessment of IL-4, IFNγ, TNFα and IL-2, mRNA production

Multiplex RT-SQ-PCR was used to assess the expression of TNFα, IL-2, IL-4 and IFNγ mRNA in the kidneys of mice with secondary systemic candidiasis. IL-2 mRNA was not detected in the kidney of infected or uninfected mice (data not shown). TNFα and IFNγ mRNA, but not IL-4 mRNA species, were produced in the kidneys on day one in mice with secondary systemic candidiasis. By day four of secondary systemic candidiasis the level of TNFα and IFNγ mRNA in the kidneys of BALB/c mice had decreased by over four fold compared to day one. In contrast, IL-4 mRNA was undetectable on day one of secondary systemic candidiasis but by day four was higher than TNFα and IFNγ mRNA.

In the kidney of mice with primary systemic candidiasis at day four only TNFα and IL-4 mRNA species were detectable. IFNγ mRNA was not detectable in the kidney of mice with primary systemic candidiasis (Figure 7.2). TNFα mRNA levels in the kidneys of BALB/c mice at day four of primary systemic candidiasis were similar to that seen in the kidneys of mice with secondary systemic candidiasis at day four. In contrast, IL-4 mRNA expression in the kidneys of mice with primary systemic candidiasis was approximately 2 fold lower than in the kidneys of mice at day four of secondary systemic candidiasis (Figure 7.2). TNFα and IL-4 mRNA but not IFNγ mRNA were detectable in the kidneys of uninfected mice (Figure 7.2). The pattern of gene expression in the kidneys of uninfected BALB/c mice and mice with primary systemic candidiasis confirmed the data obtained in chapter 6, in that, IL-4 mRNA was consistently higher than IFNγ mRNA in the kidneys of these mice. The IL-4 and IFNγ mRNA expression patterns described above were confirmed in a repeat experiment (data not shown).

Qualitative assessment of G-CSF and GM-CSF mRNA production.

Conventional RT-PCR was used for a qualitative assessment of G-CSF and GM-CSF mRNA expression in the kidney of BALB/c mice. G-CSF mRNA was not detected in the kidneys of BALB/c mice during secondary or primary systemic candidiasis. Additionally, G-CSF mRNA was not detectable in the kidneys of uninfected mice. In contrast, GM-CSF mRNA was expressed in the kidney on day one of secondary systemic candidiasis, with variable production on day four. GM-CSF mRNA was detected during primary systemic candidiasis on day four and was undetectable in uninfected mice.

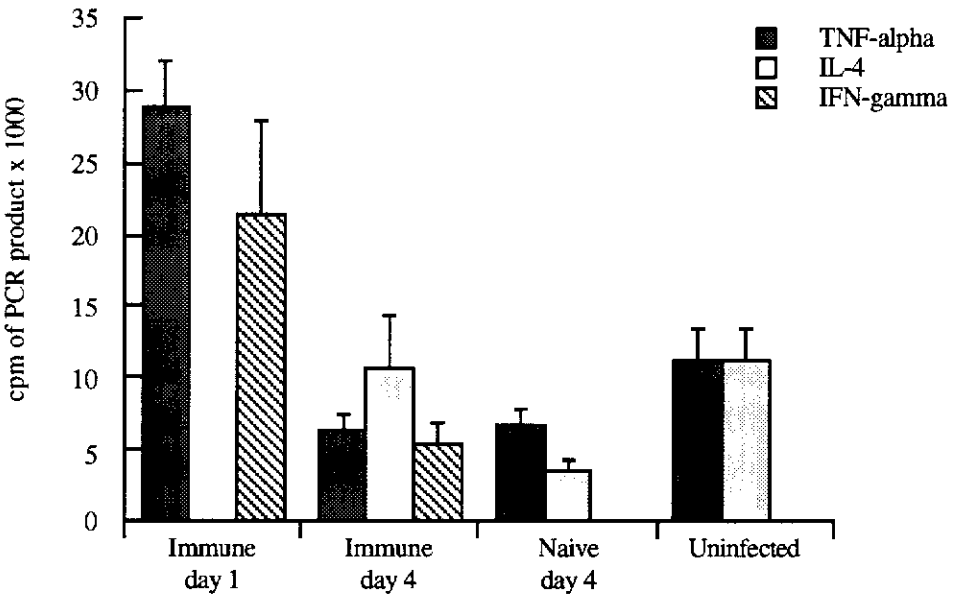

Figure 7.2 TNFα, IL-4 and IFNγ mRNA levels in the kidneys of immunised and naive BALB/c mice as assessed by multiplex SQ-RT-PCR. Immune BALB/c mice were given $1x10^5$ viable *C. albicans* blastospores *iv* and rechallenged 25 days later with $1x10^6$ viable *C. albicans* blastospores. Naive mice were given saline in the first inoculation followed by $1x10^6$ viable *C. albicans* blastospores at the time of secondary inoculation. Uninfected mice received only sterile non-pyrogenic saline. Kidneys were removed at the times indicated, after the second inoculation, and the cytokine mRNA profiles were assessed by multiplex RT-SQ-PCR. Values shown are mean of 5-6 mice per group ± SEM.

7.3.1.3 *In vivo* cytokine mRNA expression in the brain

Brain tissue was removed from infected and uninfected mice. Total RNA was extracted and used in a multiplex RT-SQ-PCR for the detection of TNFα, IL-2, IL-4 and IFNγ mRNA. Conventional PCR was exploited to assess G-CSF and GM-CSF mRNA production.

SQ-RT-PCR assessment of TNFα, IL-2, IL-4 and IFNγ mRNA production

Multiplex RT-SQ-PCR was used to assess the production of IL-2, IL-4, IFNγ and TNFα mRNA in the brain of uninfected BALB/c mice and BALB/c mice with primary and secondary systemic candidiasis. IL-2 mRNA was not detectable in the brain of BALB/c mice, whether infected or uninfected (data not shown). In initial experiments, IL-4 and IFNγ mRNA while present, was too low for accurate quantification (data not shown). TNFα mRNA was quantifiable and was highest at day one of secondary systemic candidiasis (Figure 7.3). TNFα mRNA expression levels were reduced from day 1 to day 4 of secondary systemic candidiasis. TNFα mRNA in the brain of BALB/c mice was over 3 fold higher on day four of secondary systemic candidiasis than in the brain of mice on day four of primary systemic candidiasis (Figure 7.3). TNFα mRNA levels were higher in the brain of uninfected mice, than in mice with primary systemic candidiasis, but less than was found during secondary infection.

Repeats of the multiplex SQ-PCR with IL-4 and IFNγ allowed for the measurement of IFNγ mRNA (Figure 7.4), but failed to find measurable IL-4 mRNA. Similar levels of IFNγ mRNA were detected in the brain on day one and day four of secondary systemic candidiasis compared to uninfected mice (Figure 7.4). However, IFNγ mRNA was not measurable on day four of primary systemic candidiasis.

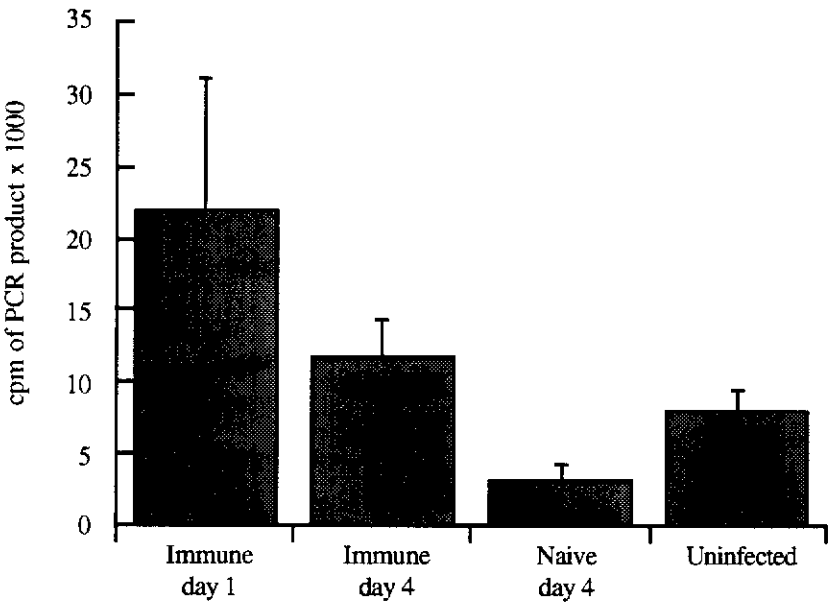

Figure 7.3 TNFα mRNA levels in the brain of immunised and naive BALB/c mice. BALB/c mice were given $1x10^5$ viable *C. albicans* blastospores *iv* and rechallenged 25 days later with $1x10^6$ viable *C. albicans* blastospores. Primary infected mice were given saline in the first inoculation followed by $1x10^6$ viable *C. albicans* blastospores at the time of secondary inoculation. Uninfected mice received non-pyrogenic saline only. Brain tissue was removed at the times indicated, after second inoculation, and the cytokine mRNA profiles were assessed by multiplex RT-SQ-PCR. Values shown are mean of 5-6 mice per group ± SEM.

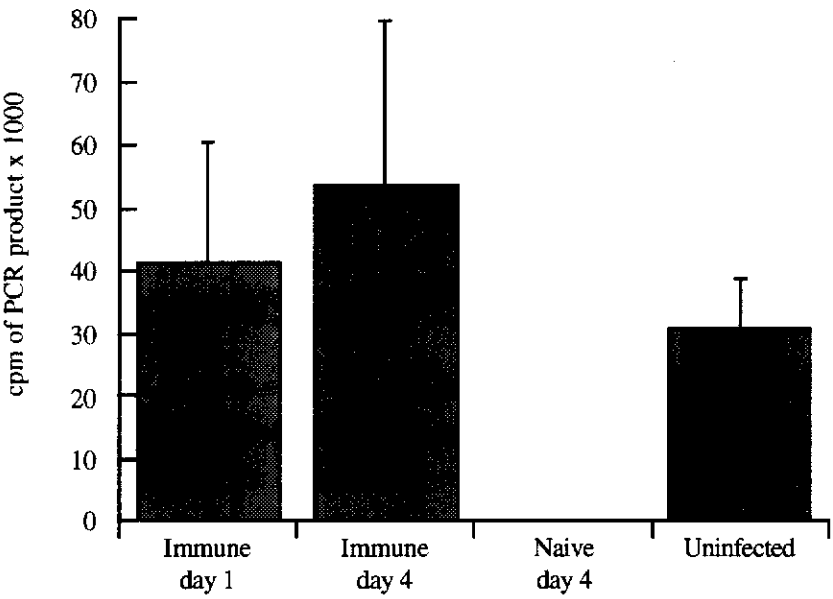

Figure 7.4 IFNγ mRNA levels in the brain of immunised and naive BALB/c mice. BALB/c mice were given $1x10^5$ viable *C. albicans* blastospores *iv* and rechallenged 25 days later with $1x10^6$ viable *C. albicans* blastospores. Primary infected mice were given saline in the first inoculation followed by $1x10^6$ viable *C. albicans* blastospores at the time of secondary inoculation. Uninfected mice received non-pyrogenic saline only. Brain tissue was removed at the times indicated, after second inoculation, and the cytokine mRNA profiles were assessed by multiplex RT-SQ-PCR. Values shown are mean of 5-6 mice per group ± SEM.

Qualitative assessment of G-CSF and GM-CSF mRNA production.

Conventional RT-PCR was used in a qualitative assessment of G-CSF and GM-CSF mRNA expression in the brain of BALB/c mice. G-CSF and GM-CSF mRNA was not detected in the brain of mice with primary or a secondary infection. Additionally G-CSF and GM-CSF mRNA was not detected in the brain of uninfected BALB/c mice.

7.3.2 Secondary systemic candidiasis in CBA mice

Immunisation of CBA mice with a sublethal dose of *C. albicans* led to significant protection from subsequent re-challenge (chapter 3). Studies were undertaken to determine the cytokine mRNA production profile of immunised mice during systemic candidiasis. Mice were immunised *iv* with $1x10^4$ viable *C. albicans* and re-challenged *iv* with $1x10^6$ viable *C. albicans* 21 days later (2.4.3). Tissues were removed, frozen in liquid nitrogen and stored at -80°C until needed. Total RNA was extracted from tissues as described (2.8.7) and the cytokine mRNA profiles determined by RT-SQ-PCR (2.9). Samples were also subjected to conventional PCR (2.8.6)

Semi-quantitative PCR was not employed for IL-2 mRNA in this set of experiments as pervious experiments had failed to find significant levels of this cytokine in primary systemic candidiasis in CBA mice and in primary and secondary systemic candidiasis in BALB/c mice. It was also decided to remove TNFα in this experiment as TNFα was detected in all tissues so far assayed in both secondary and primary infection and appeared to provide little information on tissue differences or mouse strain differences in infection.

The cytokine mRNA response to secondary infection was compared to that of uninfected mice and naive mice with primary systemic candidiasis. Uninfected and

naive mice received non pyrogenic saline at the time of immunisation and were subsequently inoculated 21 days later with non pyrogenic saline or $1x10^6$ viable *C. albicans* respectively. Cytokine profile was assessed at day one and four of secondary infection in the spleen, kidney and brain. Uninfected mice and mice with primary systemic candidiasis were assessed for cytokine production in the same organs on day four.

7.3.2.1 *In vivo* cytokine mRNA expression in the spleen

Spleens were removed from infected and uninfected mice. Total RNA was extracted and used in a multiplex RT-SQ-PCR for the detection of IL-4 and IFNγ mRNA. Conventional PCR was exploited to assess G-CSF and GM-CSF mRNA production.

SQ-RT-PCR assessment of IL-4 and IFNγ mRNA production

Multiplex RT-SQ-PCR of splenic RNA for was employed to assess IL-4 and IFNγ mRNA expression during systemic candidiasis in CBA mice. Similar levels of IL-4 to IFNγ mRNA was demonstrated in the spleen of CBA mice on day one of secondary systemic candidiasis (Figure 7.5). By day four of systemic candidiasis, levels of IFNγ mRNA in the spleen were slightly reduced. In contrast, IL-4 mRNA levels remained similar to those detected on day one. Only one naive CBA mouse survived primary infection to day four. IL-4 mRNA but not IFNγ mRNA was detected in the spleen of the remaining naive CBA mouse (Figure 7.5). Likewise, IL-4 mRNA but not IFNγ mRNA was detected in the spleen of uninfected CBA mice (Figure 7.5).

Non immunised BALB/c mice were included in this experiment as a means of comparing the results of these experiments to section 7.3.1. These mice produced only IL-4 mRNA, with IFNγ mRNA below the limit of detection, repeating the pattern

of cytokine mRNA production seen in section 7.3.1.1 and the pattern of production in the single naive CBA mouse (data not shown).

Qualitative assessment of G-CSF and GM-CSF mRNA production.

Conventional RT-PCR was used in a qualitative assessment of G-CSF and GM-CSF mRNA expression in the spleen of CBA mice. G-CSF mRNA was below the limit of detection in the spleen of CBA mice from all treatment groups. In contrast low levels of GM-CSF mRNA was detected in the spleens of CBA mice on day one of secondary systemic candidiasis. Variable production of GM-CSF was evidenced on day four of secondary systemic candidiasis. There was also variable production of GM-CSF in the spleen of uninfected mice, with barely detectable levels of GM-CSF mRNA in the remaining primary infected mouse at day four (data not shown).

7.3.2.2 *In vivo* cytokine mRNA expression in the kidneys

Kidneys were taken from infected and control mice. Total RNA was extracted and used in a multiplex RT-SQ-PCR for the detection of IL-4 and IFNγ mRNA. Conventional PCR was exploited to assess G-CSF and GM-CSF mRNA production.

SQ-RT-PCR assessment of IL-4 and IFNγ mRNA production

Multiplex RT-SQ-PCR on RNA extracted from the kidney was employed for the semi-quantification of IL-4 and IFNγ mRNA *in vivo*. IFNγ mRNA but not IL-4 mRNA was detected in the kidneys of CBA mice on day one of secondary systemic candidiasis (Figure 7.6). IFNγ mRNA levels in the kidneys of CBA were slightly reduced by day four of systemic candidiasis compared to day one. In contrast, IL-4 mRNA levels in the kidneys of CBA mice increased from being undetectable at day one to approximately one third the level of IFNγ mRNA by day four of secondary

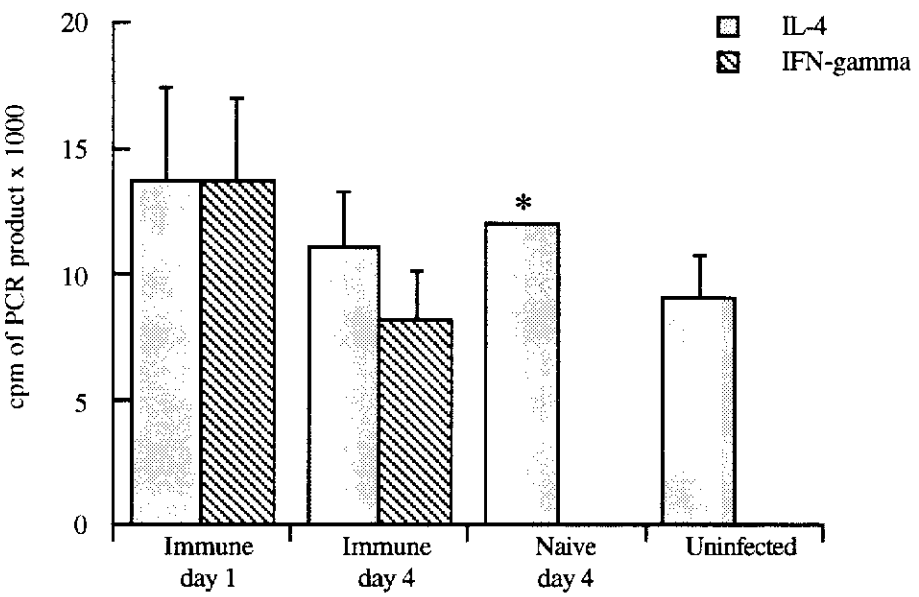

Figure 7.5 IL-4 and IFNγ mRNA production in the spleen of immunised and naive CBA mice. Immune CBA mice were given $1x10^4$ viable *C. albicans* blastospores *iv* and rechallenged 21 days later with $1x10^6$ viable *C. albicans* blastospores. Naive mice were given saline in the first inoculation followed by $1x10^6$ viable *C. albicans* blastospores at the time of secondary inoculation. Uninfected mice received non pyrogenic saline at both inoculations. Spleens were removed at the times indicated after the second inoculation and the extracted RNA used in a multiplex RT-SQ-PCR for IL-4 and IFNγ. Values are mean ± SEM of 4 to 5 mice per group.

* Only one of five mice survived primary infection with $1x10^6$ viable *C. albicans* blastospores, shown is level of IL-4 mRNA from a single mouse.

systemic candidiasis. IFNγ mRNA was below the limit of detection in the kidneys of CBA mice with primary systemic candidiasis and in the kidneys of uninfected CBA mice. Similar results were obtained in a previous experiment (data not shown). Although only one CBA mouse survived a primary infection of $1x10^6$ *C. albicans* to day four, the pattern of IL-4 and IFNγ mRNA in this mouse was identical to that demonstrated in two independent experiments in naive BALB/c mice (Figure 7.2 and data not shown). Similar results were also obtained in chapter 6, in which IL-4 was consistently higher than IFNγ mRNA in the kidneys of CBA mice with primary systemic candidiasis.

Qualitative assessment of G-CSF and GM-CSF mRNA production.

Conventional RT-PCR of RNA extracted from the kidney was used for qualitative assessment of G-CSF and GM-CSF mRNA expression. G-CSF mRNA expression was variable in the kidneys of CBA mice during secondary systemic candidiasis on day one. G-CSF was not detectable on day four of secondary infection. G-CSF mRNA was not detected in the kidneys of uninfected CBA mice, but was detectable in the kidneys of the remaining CBA mouse with primary systemic candidiasis. GM-CSF mRNA was found in the kidneys of all infected CBA mice but was not detectable in uninfected CBA mice (data not shown).

7.3.2.3 *In vivo* cytokine mRNA expression in the brain

Brain tissue was taken from infected and uninfected mice. Total RNA was extracted and used in a multiplex RT-SQ-PCR for the detection of IL-4 and IFNγ mRNA. Conventional RT-PCR was exploited to assess G-CSF and GM-CSF mRNA production.

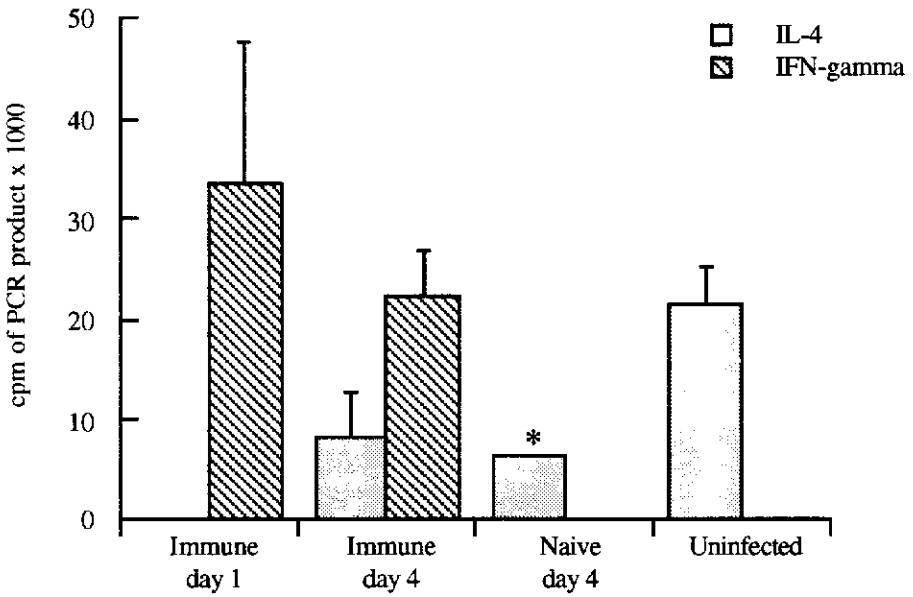

Figure 7.6 IL-4 and IFNγ mRNA production in the kidneys of immunised and naive CBA mice. Immune CBA mice were given $1x10^4$ viable *C. albicans* blastospores *iv* and rechallenged 21 days later with $1x10^6$ viable *C. albicans* blastospores. Naive mice were given saline in the first inoculation followed by $1x10^6$ viable *C. albicans* blastospores 21 days later. Uninfected mice received non pyrogenic saline at both inoculations. Kidneys were removed at the times indicated after second inoculation and the extracted RNA used in a multiplex RT-SQ-PCR for IL-4 and IFNγ. Values are mean ± SEM of 4 to 5 mice per group.

* Only one of five mice survived primary infection with $1x10^6$ viable *C. albicans* blastospores, shown is level of IL-4 and IFNγ mRNA from a single mouse.

SQ-RT-PCR assessment of IL-4 and IFNγ mRNA production

Multiplex RT-SQ-PCR was used to assess the expression of IL-4 and IFNγ mRNA in the brain of CBA mice with secondary systemic candidiasis. Cytokine production in the brain of CBA mice was consistently at the limit of detection and hence below the level of accurate quantification in this experiment. IL-4 mRNA levels appeared similar in all treatment groups (data not shown). However, IFNγ mRNA was only detectable in the brain of the remaining CBA mouse with primary systemic candidiasis (data not shown).

Qualitative assessment of G-CSF and GM-CSF mRNA production.

Conventional RT-PCR was used in a qualitative assessment of G-CSF and GM-CSF mRNA expression in the brain of CBA mice. G-CSF mRNA was detected at low levels in the brain of CBA mice on day one of secondary infection but not at day 4 of secondary infection. G-CSF mRNA was not detected in the brain of uninfected mice but was detectable in the brain of the surviving mouse with primary systemic candidiasis. GM-CSF mRNA was not detectable in the brains of mice from any treatment groups.

DISCUSSION

Immunisation of BALB/c and CBA mice with a sublethal inoculum of *C. albicans* led to significant protection from re-challenge with an increased dose of *C. albicans* (chapter 3; Table 3.1 and 3.2). Similar results were obtained by Bretscher *et al* (1992) when they demonstrated that immunisation of mice with a small inoculum of *L. major* induces a switch from non-protective humoral responses to protective cell mediated responses. In a murine model of systemic candidiasis Romani *et al* (1991b) demonstrated similar results to those described by Bretscher *et al* (1992), in that immunisation of mice with an avirulent strain of *C. albicans* protected mice from subsequent infection with a virulent strain of *C. albicans* by a Th1 or cell mediated response. However, the data obtained by Romani *et al* (1991b) is contradicted by earlier papers which suggest that T-cells play no role in increased resistance to secondary systemic candidiasis in their model (Bistoni *et al.*, 1988; Vecchiarelli *et al.*, 1988; discussed in detail page 98. Romani *et al* (1991b) did not study the *in vivo* production of cytokine during secondary systemic candidiasis. Consequently, cytokine mRNA profiles were studied in mice with secondary systemic candidiasis to determine if Th1 responses were associated with the increased resistance to systemic candidiasis in this model of systemic candidiasis. Additionally, as previous studies had shown that cytokine gene expression varied from organ to organ during primary systemic candidiasis (chapter 6), the cytokine responses in the spleen, kidney and brain of BALB/c and CBA mice were monitored to determine if this also occurred during secondary systemic candidiasis.

In the spleen of BALB/c mice during secondary systemic candidiasis IFNγ mRNA levels were marginally higher than those of IL-4 mRNA (Figure 7.1). In contrast, IFNγ mRNA was not detected in the spleen of BALB/c mice with primary systemic candidiasis and was lower than IL-4 mRNA in uninfected mice. A similar response was seen in CBA mice, in that only mice with secondary systemic candidiasis

expressed detectable levels of IFNγ mRNA (Figure 7.5). These results confirm those detailed in chapter 5 in that IL-4 mRNA was consistently higher than levels of IFNγ mRNA levels in the spleens of mice with primary systemic candidiasis (Table 5.1). These experiments suggest a Th2 like response in the spleen of BALB/c and CBA mice with primary systemic candidiasis which switches to a more Th0 like response during secondary systemic candidiasis. The increased IFNγ mRNA demonstrated in the spleen of both healer BALB/c and non-healer CBA mice is likely to inhibit Th2 responses, or result from lower Th2 responses. This may be of particular importance as inhibition of Th2 (Romani *et al.*, 1992c; Puccetti *et al.*, 1994; Romani *et al.*, 1994b), but not Th1 (Romani *et al.*, 1992a; Romani *et al.*, 1992b) responses protect mice from primary systemic candidiasis. Therefore, any inhibition of a Th2 response in the spleen is likely to be associated with the increased survival of mice with secondary as compared to primary systemic candidiasis. Additionally, these data reinforce earlier data (chapter 5 and 6) that demonstrated there was little difference in cytokine gene expression between CBA and BALB/c mice.

The reversal of the IFNγ/IL-4 mRNA ratio in BALB/c and CBA mice during secondary systemic candidiasis compared to uninfected mice and mice with primary systemic candidiasis suggests an active response in these mice to *C. albicans*. However, particularly in BALB/c mice, the production of cytokine mRNA species such as TNFα and IL-4 mRNA were lower than those of uninfected mice. This suggests that while cells are primed within the spleen, they have suppressed or static cytokine production. These data are similar to data obtained in chapter 5, which demonstrated that cytokine mRNA expression in the spleens of uninfected mice was consistently higher than in infected mice. Hence, there appears to be a *C. albicans* mediated suppression of cytokine expression in the spleen of infected mice. However, the cytokine gene suppression demonstrated in the spleen was focused to this site, as cytokine mRNA was induced at the sites of infection in the brain (chapter 6; Figures 6.1, 6.2, 6.3) and kidney (Figure 7.2 and 7.6). Additionally, the induction of IFNγ

mRNA in the spleen of mice with secondary systemic candidiasis suggests that activation is occurring at this site. Consequently, priming of the immune response may occur in the spleen. However, active cytokine mRNA expression at this site was suppressed. This may be a result of activated T-cells migrating out of the spleen to peripheral sites as others have shown that the percentage of T-cells decreases in the spleen during systemic candidiasis (Costantino, 1995). Alternatively, this suppression may serve as a mechanism of ensuring inflammatory responses are focused at the site of infection, such as the kidneys, rather than the spleen. Hence, measurement of cytokine gene expression in the spleen does not always reflect the cytokine gene expression at the site of infection. Additionally, the reduced cytokine mRNA expression in the spleen may be a normal physiological response to infection, rather than a *C. albicans* induced immunosuppression.

More IFNγ mRNA was induced in the kidneys of mice with secondary systemic candidiasis than in the spleen (Figure 7.2 and 7.6). This was seen in the kidneys of both healer and non-healer mice and was more evident on day one of secondary systemic candidiasis than on day four. On day one of secondary systemic candidiasis the high IFNγ mRNA levels in the kidney were seen in conjunction with an absence of detectable IL-4 mRNA. In contrast, uninfected mice and mice with primary systemic candidiasis did not produce detectable levels of IFNγ mRNA in these experiments. Additionally, in previous experiments (chapter 5 and 6) IL-4 mRNA levels were consistently higher than IFNγ mRNA in the kidneys and the spleen of uninfected mice and mice with primary systemic candidiasis at all the time points measured. By day four of secondary systemic candidiasis the pattern of cytokine production in the kidneys had reverted to a more Th0 pattern, particularly in BALB/c mice, with increased IL-4 mRNA and decreased IFNγ mRNA.

The lack of cytokine expression in the kidneys of mice with primary systemic candidiasis, coupled with a predominantly neutrophil infiltrate was associated with

failure of the organ to resolve infection, particularly in CBA mice (chapter 3). In contrast, during secondary systemic candidiasis the Th1 cytokine profile in the kidneys was associated with decreased *C. albicans* colonisation in the kidneys of mice with secondary systemic candidiasis compared to primary systemic candidiasis (chapter 3). However, of interest, is the finding that cytokine mRNA was reduced from day one to day four in the kidneys of BALB/c and CBA mice. This reduction in cytokine mRNA, and in particular IFNγ mRNA, was in conjunction with increased IL-4 mRNA expression. In BALB/c mice the reduction in IFNγ mRNA expression and increase in IL-4 mRNA from day one to day four was seen in conjunction with a 10 fold decrease in the level of *C. albicans* colonisation of the kidney (chapter 3). In contrast, the level of yeast colonisation increased 10 fold in CBA mice from day one to day four. Consequently, while both BALB/c and CBA mice had similar patterns of IFNγ and IL-4 mRNA expression in the kidneys during secondary systemic candidiasis, in CBA mice this was not sufficient to limit the increase in yeast load. Additionally, immunised CBA mice, while reducing the level of colonisation in the kidney compared to naive mice, still had a higher yeast load in this organ than either naive or immune BALB/c mice (chapter 3). Consequently, while Th1 cytokines may increase the candidacidal activity of neutrophils (Djeu *et al.*, 1986; Tansho *et al.*, 1994) the increased expression of IFNγ mRNA in the kidneys of CBA mice was insufficient to compensate for any underlying defect in these mice. Increased resistance in BALB/c mice compared to CBA mice may be due to the increased numbers of macrophages in the kidneys of BALB/c mice.

Cytokine gene expression was low in the brain in the experiments detailed in this chapter (Figures 7.3 and 7.4). The low cytokine mRNA levels detected were most likely a reflection of the reduced abscess formation in the brain of mice with secondary systemic candidiasis.

It appears likely from these studies that protection from systemic candidiasis by immunisation is mediated, at least in part, by a shift to a Th1 like phenotype and a shift away from a Th2 like phenotype in both BALB/c and CBA mice. This conclusion is supported by the findings of other studies in which Th1 responses are protective in *C. albicans* infections (Romani *et al.*, 1991b) and in other infectious disease models (Scott *et al.*, 1988; Scott *et al.*, 1989). The mechanism for the induction of a Th1 like response to secondary infection in the absence of any apparent Th1 response to primary infection is unclear. However, the response to infection with different doses of *C. albicans* follows closely the phenomenon of low zone tolerance described by Parish (1972) and others (Parish and Liew, 1972; Stumpf *et al.*, 1977; Bretscher *et al.*, 1992). This describes the phenomenon that immunisation with low Ag dose stimulates preferentially a Th1 or cell mediated response and inhibits a Th2 or humoral response. At high Ag doses the reciprocal occurs with humoral responses dominating. In the model of systemic candidiasis described in this Thesis, an initial high primary inoculum led to cytokine response that where, in general, characterised by higher IL-4 mRNA than IFNγ mRNA. When mice were immunised with a smaller sublethal inoculum of *C. albicans* the predominant response was Th1 like, in that IFNγ mRNA predominated over IL-4 mRNA. Hence the phenomenon of low zone tolerance described by Bretscher *et al* (1992) in leishmaniasis also occurs in systemic candidiasis.

In conclusion, this chapter has demonstrated the increased resistance to systemic candidiasis of immune mice (chapter 3) was associated with expression of the Th1 cytokine IFNγ mRNA and inhibition of IL-4 mRNA. BALB/c and CBA mice made similar responses at the level of cytokine mRNA expression to both primary and secondary systemic candidiasis. Therefore, these results reinforce the conclusions of chapter 5 and 6, that no overt difference exist between cytokine mRNA expression in CBA mice compared to BALB/c mice. Since immunised CBA mice remain more sensitive to systemic candidiasis than naive BALB/c mice, the induction of a Th1 like

response to secondary systemic candidiasis was insufficient to compensate for the primary defect in CBA mice. Therefore, T-cell responses, while apparently present, do not play a major role in determining the resistance phenotypes of the CBA and BALB/c mice.

CHAPTER EIGHT

GENERAL DISCUSSION

This study has characterised a model of systemic candidiasis with the *C. albicans* strain KEMH5 obtained from a patient with vulvo-vaginal candidiasis. Systemic candidiasis induced by this strain of *C. albicans* was similar to that described for other murine models of systemic candidiasis (Hurley and Fauci, 1975; Hector *et al.*, 1982; Ashman and Papadimitriou, 1987; Ashman *et al.*, 1993). In this model, CBA mice consistently developed more severe systemic disease than comparably treated BALB/c mice, as measured by greater tissue colonisation, histopathological damage to affected organs and higher mortality. In both CBA and BALB/c mice yeast colonisation was greatest in the kidney followed by the brain. Other organs, the spleen, liver, lungs and heart, had yeast infiltration that was detectable by quantitative yeast culture but failed to develop obvious lesions when assessed by histology.

A sensitive and robust RT-SQ-PCR was developed to determine if cytokine gene expression, during infection, could account for the differences in the sensitivity of BALB/c and CBA mice to systemic candidiasis. There was no evident difference in the pattern of cytokine transcription in lymphoid or non-lymphoid organs between CBA and BALB/c mice that could be used to explain the increased sensitivity to infection of CBA mice compared to BALB/c mice. The consistent demonstration from these studies was that CBA mice expressed higher levels of most cytokine mRNA species compared to that of comparably treated BALB/c mice. This increased cytokine mRNA expression in CBA mice was likely due to the increased yeast load in these mice and not a causative feature in their decreased resistance.

This study has demonstrated a difference in the expression of cytokine mRNA in the brain compared to the kidney during systemic candidiasis. Rapid clearance of yeast in

the brain was associated with the presence of activated macrophages and or microglia (chapter 3) and the expression of cytokine mRNA species consistent with a Th1 response. In the kidneys poor clearance and increased yeast load was associated with minimal cytokine mRNA expression and a predominantly neutrophil infiltration. In the kidneys of resistant BALB/c mice, F4/80 and Ia positive cells were more prevalent than in CBA mice. This was apparent during primary and secondary infection. This data along with the observations of high numbers of activated macrophages in the brain suggests that macrophages are crucial to the clearance of infection. This hypothesis is supported by observations from other studies in which resistance to systemic candidiasis is associated with replacement of the renal PMN infiltrate with a mononuclear infiltrate (Romani *et al.*, 1992c; Puccetti *et al.*, 1994). Therefore, BALB/c mice appear to be more resistant, at least in part, due to an ability to recruit more activated F4/80$^+$ cells to the primary target organ of *C. albicans*.

There was no difference in the early pattern of cytokine mRNA expression in the spleen of infected BALB/c and CBA mice. Mice with primary systemic candidiasis predominantly expressed more IL-4 mRNA than IFNγ. In some experiments IL-4 mRNA expression was to the exclusion of IFNγ mRNA. In the later stages there was a switch to greater IL-4 mRNA expression in the spleen of CBA mice. The timing of this switch to a more "Th2 like" response in the spleens of CBA mice was consistent with the onset of mortality. A subsequent experiment demonstrated that decreased IFNγ mRNA was associated with increased mortality to chronic systemic candidiasis. Therefore, reduction in IFNγ mRNA or induction of a Th2 like response in CBA mice was associated with increased mortality to chronic systemic candidiasis. However, it appears likely that IFNγ has different roles in acute and chronic systemic candidiasis as neutralisation of IFNγ increased the resistance to both strains of mice to acute primary systemic candidiasis.

The switch to a "Th2 like" response in the spleens of CBA mice was not apparent until day five of primary systemic candidiasis. Differences in the resistance of CBA and BALB/c mice to systemic candidiasis were apparent within 24 hours of infection. Therefore, this response appears to be a sequelae to an inability to clear the infection, possibly due to increased Ag load in the CBA mice (Parish, 1972; Bretscher *et al.*, 1992; Secrist *et al.*, 1995). However, while the immune deviation does not appear to be the primary difference in the resistance phenotypes, induction of a Th2 response is likely to have detrimental effects on resistance to infection, as Th1 responses may be required for optimal macrophage activation. Hence, the induction of a Th2 response may explain the increased mortality and yeast load in the kidneys of CBA mice in chronic infection.

Inhibition of the PGE_2 production by treatment with indomethacin did not increase the resistance of CBA and BALB/c mice to primary systemic candidiasis. Indeed inhibition of PGE_2 production increased the sensitivity of both CBA and BALB/c mice to systemic candidiasis. Additionally, in CBA mice the increased sensitivity to chronic systemic candidiasis, induced by indomethacin treatment, was associated with a reduction in IFNγ mRNA production. Hence, the reduction of cytokine mRNA seen in the spleen and kidneys during primary systemic candidiasis was not due to excess PGE_2 production. Finally, the increased IL-4/IFNγ mRNA ratio noted in the spleen of CBA mice during chronic infection does not appear to be a result of excessive PGE_2 production. Therefore, it is remains unclear why the cytokine mRNA production was suppressed in the spleen of infected mice. However, it is possible this was related to a reduction in total T-cell numbers in the spleen (Costantino, 1995a).

Secondary infection in both strains of mice was characterised by increased resistance to disease, as determined by tissue colonisation levels and histology. Increased resistance in both CBA and BALB/c mice was associated with an increase in IFNγ mRNA expression and a decrease in the expression of IL-4 mRNA. This was

evidenced in the spleen and kidneys of infected mice. Cytokine production was low in the brain during secondary systemic candidiasis, due, most likely, to reduced abscess formation. Hence, these studies have confirmed the findings of other studies, in that, immunisation of mice with *C. albicans* can increase resistance to systemic candidiasis (Hector *et al.*, 1982) and that the increased resistance appears to be due to production of a Th1 response (Romani *et al.*, 1991b). Finally, this data recapitulates that seen in leishmaniasis (Bretscher *et al.*, 1992), in that inoculation with a sublethal dose of *C. albicans* was able to stimulate a Th1 like response.

Data from this study strongly suggests that T-cells do not play a major role in resistance to primary and secondary systemic candidiasis. Additionally, differences in the resistance phenotypes of CBA and BALB/c mice was not due to differences in T-cell function. Differences in resistance to systemic candidiasis were apparent as early as day one of infection. No differences were found in the early expression of IL-1α, IL-2, IL-3, IL-4, IFNγ, TNFα, M-CSF, GM-CSF and G-CSF mRNA species between CBA and BALB/c mice in any organ studied. There was no evidence that CBA mice were more sensitive to acute systemic candidiasis due to a Th2 pattern of cytokine production, as has been shown with sensitive mice in other models of systemic candidiasis (Romani *et al.*, 1993). T-cells were not prevalent in infected tissue but were more prevalent in the kidneys that the brain. However, the kidneys consistently had greater levels of yeast colonisation than the brain, suggesting that the increased T-cell numbers in the kidney had little effect on protecting this tissue for infection. Inhibition of IFNγ increased, not decreased, resistance to primary systemic candidiasis. Finally, immunised CBA mice, even with a Th1 pattern of cytokine mRNA expression, remained more sensitive to infection than naive BALB/c mice.

The hypotheses that T-cells play little role in the resistance to systemic candidiasis is supported by evidence from other studies. Inhibition of IFNγ does not alter the outcome of primary systemic candidiasis in other murine models (Romani *et al.*,

1992a; Romani *et al.*, 1992b). Administration of recombinant murine IFNγ increased the mortality of naive Swiss Webster, CBA and BALB/c mice and increased the yeast load in tissues of naive A/J and CBA mice (Garner *et al.*, 1992). Studies in nude and thymectomised mice have not demonstrated increased sensitivity to systemic candidiasis (Cutler, 1976; Rogers *et al.*, 1976; Giger *et al.*, 1978; Tabeta *et al.*, 1984). Depletion of $CD4^+$ and $CD8^+$ cells does not alter the out come of primary infection (Cenci *et al.*, 1989; Romani *et al.*, 1992b). The evidence that T-cells are important because resistant mice have increased DTH responses compared to sensitive mice is circumstantial (Ashman, 1990) or contradictory (Hector *et al.*, 1982; Ashman, 1990). Studies have linked increased resistance to secondary systemic candidiasis to a Th1 responses (Romani *et al.*, 1991b; Romani *et al.*, 1993) and shown that T-cells are involved in resistance to secondary infection (Cenci *et al.*, 1989). However, in earlier studies using the same or a similar systems, resistance to secondary infection was mediated by macrophages (Vecchiarelli *et al.*, 1988) and was independent of T-cells (Bistoni *et al.*, 1988). Finally, even in immunised mice, depletion of $CD4^+$ cells does not affect resistance to infection (Cenci *et al.*, 1989). The hypothesis that T-cells are not required for resistance to systemic candidiasis is not only evident in murine models. AIDS patients frequently develop oral and oesophageal candidiasis. However, these patients rarely develop systemic candidiasis. Hence T-cells appear to be important in human cutaneous candidiasis but not in systemic candidiasis.

In contrast to T-cells, there was substantial evidence that macrophages have a major role in resistance to systemic candidiasis. Activated macrophages were present in tissues that resisted infection. Activated macrophages were more prevalent in the kidneys of resistant BALB/c mice than in the kidneys of sensitive CBA mice. This was evidence during both primary and secondary systemic candidiasis. In fact macrophages appeared to be increased in the kidneys of BALB/c mice with secondary systemic candidiasis compared to primary infection. M-CSF, a cytokine that affects macrophages, not neutrophils, and increases their activation (Unanue, 1993), was the

only CSF mRNA that was consistently detected in mice with systemic candidiasis. Surprisingly, cytokines that affect the development and activation of neutrophils and macrophages, such as, G-CSF, GM-CSF and IL-3 were difficult to detect, or not present, in every tissue tested.

Other studies also suggest that macrophages are important in resolution of systemic candidiasis. M-CSF protein was found in the tissues of mice with systemic candidiasis (Cenci *et al.*, 1991). M-CSF increases the candidacidal activity of macrophages (Karbassi *et al.*, 1987; Wang *et al.*, 1989b). M-CSF was protective when given to mice with systemic candidiasis (Cenci *et al.*, 1991). Macrophages were increased and T-cells decreased in the spleens of mice with systemic candidiasis (Costantino, 1995a). Macrophages have been shown to be the primary cell responsible for the increased resistance to infection demonstrated by immunised mice (Bistoni *et al.*, 1988; Vecchiarelli *et al.*, 1988). Macrophages from sensitive mice are less candidacidal than those of resistant mice (Romani *et al.*, 1994b). Finally, depletion of macrophages was associated with increased sensitivity to systemic candidiasis (Qian *et al.*, 1994). In contrast, depletion of T-cells did not affect resistance to systemic candidiasis unless IFNγ was also depleted (Romani *et al.*, 1992a; Romani *et al.*, 1992b).

Therefore, it appears likely from this study and from data obtained in other studies that macrophages are crucially important to the resolution of systemic candidiasis. However, this is in contradiction to a number of studies that have shown that Th1 responses are protective and a Th2 response are non-protective in murine models of systemic candidiasis. These data appear to contradict the assumption that T-cells play little role in resistance to systemic candidiasis. However, it is possible that Th1 and Th2 responses in mice with systemic candidiasis are a result of macrophage action and not T-cell action. Macrophages produce both IL-10 and IL-12 (Unanue, 1993), cytokines that promote a Th2 and a Th1 phenotype respectively (Howard *et al.*, 1993).

Additionally, these so called Th1 and Th2 cytokines may have their principal effects on macrophages.

Th2 cytokines have profound inhibitory effects on the action of macrophages (Cunha *et al.*, 1992; Gazzinelli *et al.*, 1992; Oswald *et al.*, 1992). Both IL-4 and IL-10 inhibit IFNγ activated macrophage killing of *C. albicans* by reducing NO production (Cenci *et al.*, 1993; Romani *et al.*, 1994b). This is reflected *in vivo* where neutralisation of IL-4 and IL-10 protects mice from systemic candidiasis (Romani *et al.*, 1992c; Puccetti *et al.*, 1994; Romani *et al.*, 1994b), while administration of IL-4 or IL-10 increases sensitivity to infection (Tonnetti *et al.*, 1995). In contrast, the effects of IFNγ on resistance to systemic candidiasis are equivocal at best (Garner *et al.*, 1992; Romani *et al.*, 1992a; Romani *et al.*, 1992b; Kullberg *et al.*, 1993).

These data suggest that macrophages can have a profound effect on resistance to systemic candidiasis. This can be due to the direct candidacidal activity of macrophages. Alternatively, macrophages could affect the development of Th1 and Th2 responses by the action of macrophage derived IL-10 and IL-12. Therefore macrophage and not T-cell derived cytokines may induce either a Th1 or a Th2 response. Interestingly, IL-12, a macrophage derived cytokine that stimulates a Th1 response may be more positively linked to resistance than IFNγ (Romani *et al.*, 1994a). Resistance to secondary infection may be a combination of the enhanced Th1 response as demonstrated in this and other studies (Romani *et al.*, 1993) and the effects on macrophages of IFNγ or the lack of negative effects of IL-4 and IL-10. It is interesting to note that macrophages need cytokines to become maximally candidacidal and that this increased activation takes longer to develop than in cytokine activated neutrophils (Calderone and Sturtevant, 1994). These data may explain the equivocal results obtained when IFNγ is depleted in acute infection (Garner *et al.*, 1992; Romani *et al.*, 1992a; Romani *et al.*, 1992b; Kullberg *et al.*, 1993). If activated macrophages are not yet present the reduction in IFNγ may have little effect. However, in the

chronic infection and during secondary infection IFNγ may be critical in ensuring increased activity of these cells.

Therefore the appearance of a Th1 or Th2 response may be coincidental to T-cell action. However, the effect of macrophage derived cytokines could perpetuate T-cell responses. Manipulation of Th1 or Th2 cytokines, a method that has been used to determine the importance of these cytokine, could alter the disease course by by-passing macrophage derived cytokines. It is interesting to note than even in Leishmaniasis, a system with a well defined difference in T-cell phenotype (Heinzel *et al.*, 1989; Scott *et al.*, 1989; Scott, 1991; Scott and Kaufmann, 1991), macrophage differences appear before T-cell difference in sensitive and resistance mice (Sunderkotter *et al.*, 1993). Additionally, IL-12 is able to drive a curative response to leishmaniasis (Sypek *et al.*, 1993). As macrophages can produce both IL-10 and IL-12 they are themselves capable of diverting the immune response. Hence, the response of T-cells when measured *in vitro* or *in vivo* may be due to the effects of macrophages not T-cells. T-cell derived cytokines may exacerbate or enhance resistance to infection, however the direction of the response may be macrophage driven.

SUGGESTIONS FOR FUTURE INVESTIGATION

The role that Th1 and Th2 responses play in the resistance to infection has been an area of considerable interest since the initial characterisation of Th1 and Th2 cells by Mosmann *et al.* (1986). Data from this and other studies suggest that immune responses to infectious diseases may not fit the Th1/Th2 paradigm as well evidence obtained from diseases such as leishmaniasis suggest. This study has demonstrated that IFNγ may have different roles in the acute and chronic stages of systemic candidiasis. It would be interesting in future studies to further define the role of IFNγ at different stages of infection. Depletion of IFNγ, by administration of mAb, in the acute and the chronic infection may have different effects on disease resistance.

This study has failed to find substantial evidence for T-cell mediated resistance to systemic candidiasis. However, the was a significant amount of evidence to suggest that macrophages play a significant role in the resolution of systemic candidiasis. Further, studies would focus on the effects of macrophages in resistance to infection and determine if macrophages from healer BALB/c mice were intrinsically more candidacidal than macrophages from non-healer CBA mice. Additionally, it would be interesting to deplete splenic macrophage populations, by administration of toxic silica or liposomes containing dichloromethyl diphosponate, to determine if macrophage contributed to the development of Th phenotypes in infected mice .

While this study found minimal evidence of T-cell involvement, at least in primary systemic candidiasis, T-cell responses may be best characterised to a specific Ag. Heat shock proteins are known to be T-cell targets in both *C. albicans* infection and in other diseases. To this end, approximately 10% of the *C. albicans* HSP60 gene has been sequenced. Completion of the sequencing will allow for the cloning of this gene and expression of HSP60 in an expression vector. Further studies would concentrate on the T-cell reactivity, if any, to this protein and characterisation of T-cell epitopes.

Finally, immunohistochemistry would be used to determine the kinetics of immune cell infiltration into infected tissues of both CBA and BALB/c mice. Further evidence of macrophage infiltration would suggest the use of macrophage depletion experiments as described above.

APPENDIX

This appendix has been added to address the concerns of two examiners. Dr Miles Bemean has requested that reference to the immunohistochemistry of the kidney be excluded from the Thesis. The examiner has stated concerns regarding the interpretation of this data as many of the cells within the renal abscesses maybe dead and hence not expressing specific Ag. Statements regarding the periphery of the abscess may be suspect due to the presence of endogenous peroxidase activity in the kidney which could mask the presence of specific cells. Therefore, the Figures relating to this series of experiments have been removed from the body of the Thesis and are appended in this appendix.

It was not felt that the data could be excluded from reference as the data was commented upon favourably by Professor John Penhale. In addition, the examiner Dr Patricia Price requested a summary table of the immunohistological data be included in the Thesis. Therefore it was felt necessary, in order to be fair to Prof J. Penhale and Dr P. Price, to remove the Figures to an appendix. In addition the Tables, as requested by Dr P. Price, have been placed in the appendix to accommodate the concerns of both Dr M. Bemean and Dr P. Price (Table A.1 and A.2). Any discussion of the data, within the body of the thesis, contained in these Figures and in the Tables (as requested by Dr P. Price), must therefore be considered as preliminary only. Reference to the preliminary nature of this data has been added to the body of the Thesis (page 84).

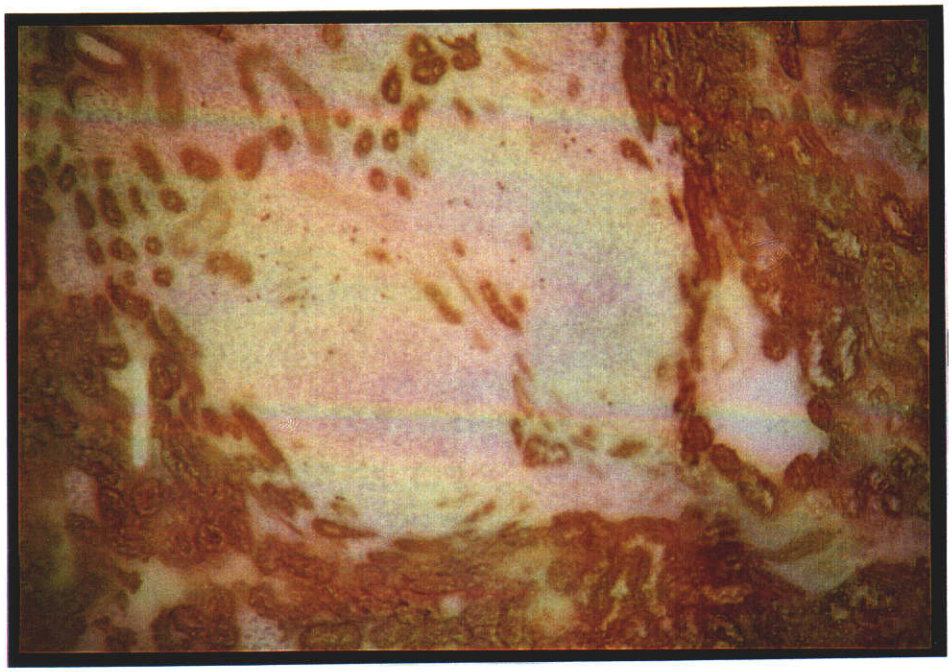

Figure A.1 Photomicrograph (x100): Control staining using sheep anti-rat secondary Ab only. Photomicrograph shows an abscess in the kidney of a CBA mouse on day four of primary systemic candidiasis. Kidney tissue has considerable endogenous peroxidase activity. Endogenous peroxidase activity could not be removed even with extensive hydrogen peroxidase treatment. However, abscesses within the kidney, shown here, do not have endogenous peroxidase activity. This allows for evaluation of immunohistological staining within the abscess. Minimal non-specific Fc staining was seen in abscesses in the kidney.

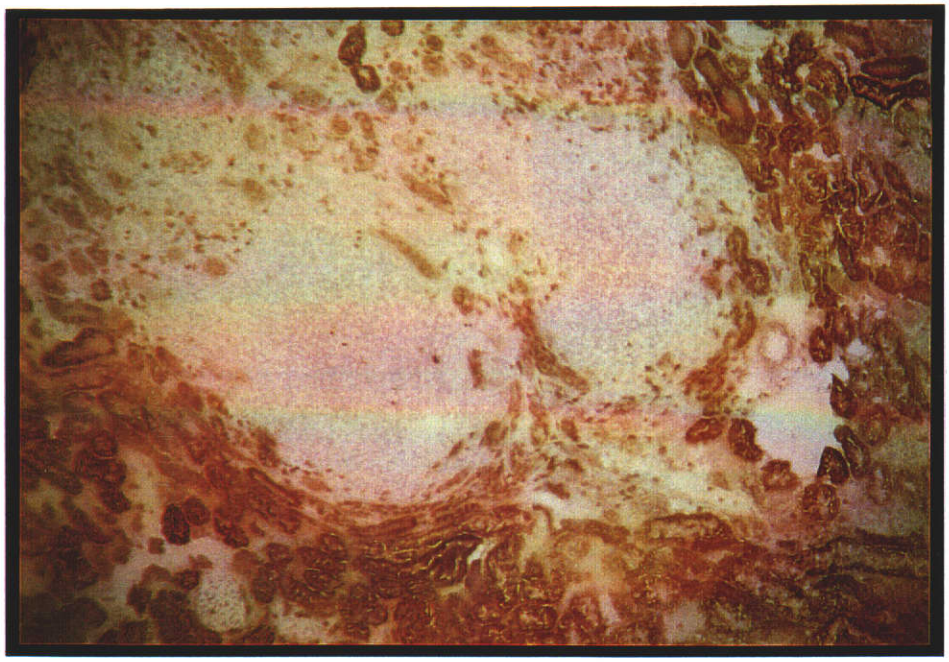

Figure A.2 Photomicrograph (x100): CBA kidney tissue stained for CD3 expression. Photomicrograph shows an abscess in the kidney of a CBA mouse on day four of primary systemic candidiasis. CD3 staining in the abscess was similar to the non-specific Fc staining seen in Figure A.1. $CD3^+$ cells can be seen in the parenchyma surrounding the abscess.

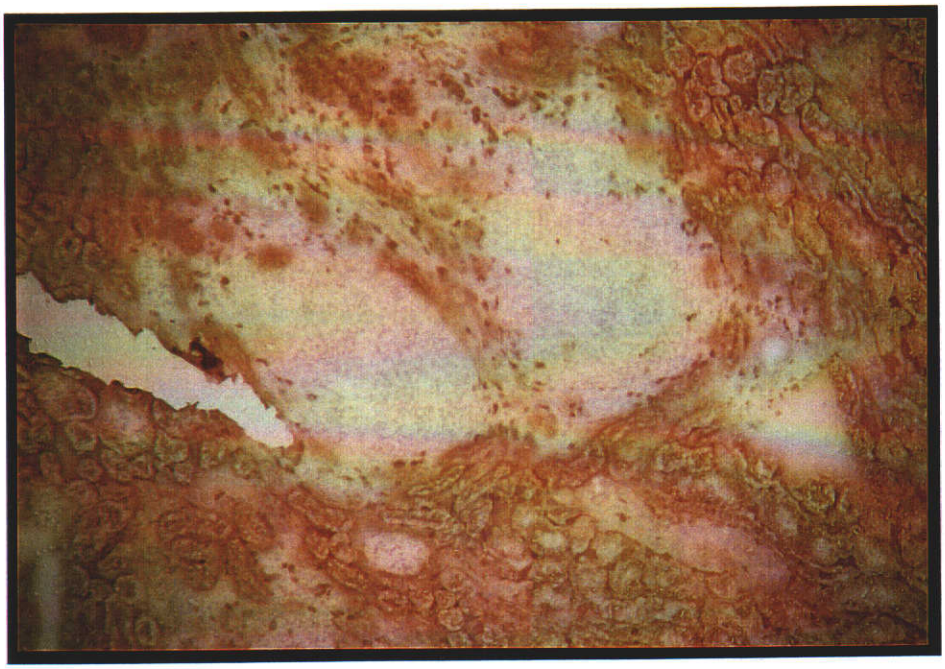

Figure A.3 Photomicrograph (x100): CBA kidney tissue stained for CD5 expression. Photomicrograph shows an abscess in the kidney of a CBA mouse on day four of primary systemic candidiasis. CD5 staining in the abscess was similar to the non-specific Fc staining seen in Figure A.1. Considerable numbers of $CD5^+$ cells can be seen at the periphery of the abscess and in the surrounding parenchyma. CD5 expression in this tissue was typical, and was, in general, higher than CD3 staining as seen in Figure A.2.

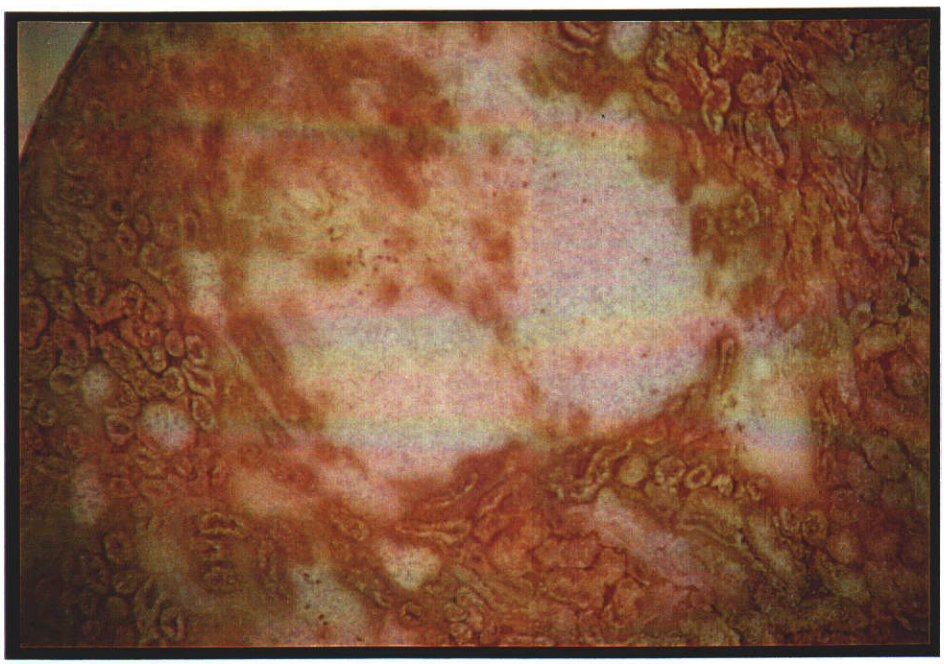

Figure A.4 Photomicrograph (x100): CBA kidney tissue stained for Ser-4 expression. Photomicrograph shows an abscess in the kidney of a CBA mouse on day four of primary systemic candidiasis. Ser-4^+ cells can not be seen in the abscess or surrounding tissue.

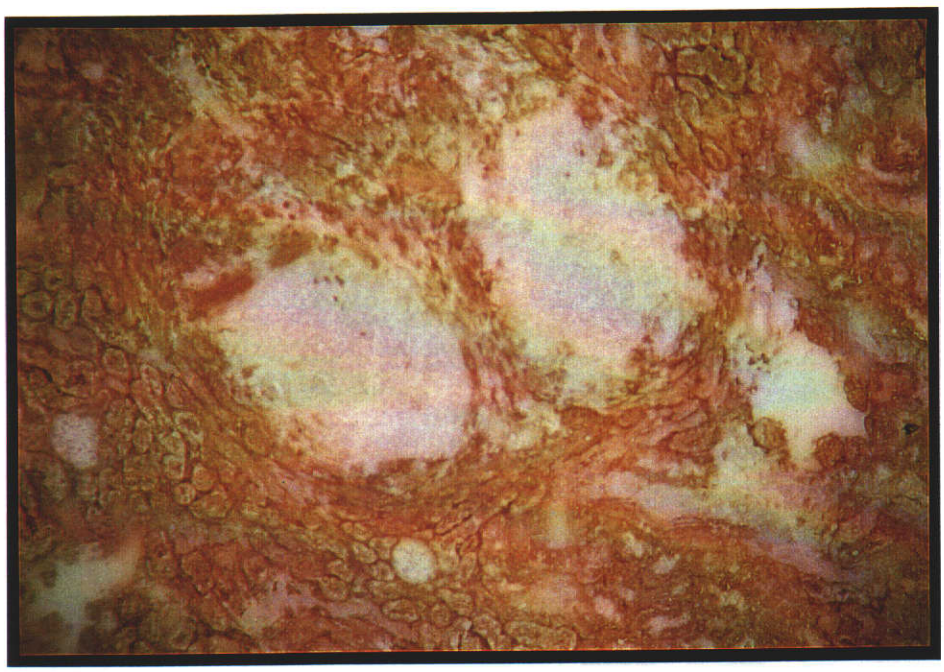

Figure A.5 Photomicrograph (x100): CBA kidney tissue stained for F4/80 expression. Photomicrograph shows an abscess in the brain of a CBA mouse on day four of primary systemic candidiasis. Very few cells expressing F4/80 were seen in the kidneys of CBA mice with primary or secondary systemic candidiasis.

Figure A.6 Photomicrograph (x100): CBA kidney tissue stained for CD11b (Mac-1) expression. Photomicrograph shows an abscess in the kidney of a CBA mouse on day four of primary systemic candidiasis. CD11b is expressed at high levels throughout the abscess which can be seen at the centre of the photomicrograph.

	CBA mice-primary systemic candidiasis		CBA mice-secondary systemic candidiais	
	Brain	Kidney	Brain	Kidney
Abscess formation	++	+++	++	++
Yeast load	++	+++	+	++
$CD3^+$ cell infiltration	±	+	±	+
$CD4^+$ cell infiltration	±	±	±	-
$CD5^+$ cell infiltration	±	+	±	+
$CD8^+$ cell infiltration	±	+	±	+
$F4/80^+$ cell infiltration	++	+±	++±	+±
Mac-1^+ cell infiltration	++±	+++	+++	+++
Ser-4^+ cell infiltration	-	-	-	-
Class II^+ cell infiltration	+	+	+	+

Table A.1 Histology and immunohistology of the brain and kidney of CBA mice on day 4 of primary and secondary systemic candidiasis.

	BALB/c mice-primary systemic candidiasis		BALB/c mice-secondary systemic candidiais	
	Brain	Kidney	Brain	Kidney
Abscess formation	+	++	±	-
Yeast load	+	++	±	-
$CD3^+$ cell infiltration	±	+	-	±
$CD4^+$ cell infiltration	±	-	-	-
$CD5^+$ cell infiltration	±	+	-	±
$CD8^+$ cell infiltration	±	±	-	±
$F4/80^+$ cell infiltration	+	+	++	++
Mac-1^+ cell infiltration	+	+	+	+±
Ser-4^+ cell infiltration	-	-	-	-
Class II^+ cell infiltration	++	++	++	++±

Table A.2; Histology and immunohistology of the brain and kidney of BALB/c mice on day 4 of primary and secondary systemic candidiasis.

Table A.1 and A.2: Data shown is a summary of the data contained in section 3.5.1.1 to 3.5.1.2 for conventional histology and section 3.5.3 for immunohistochemistry. Key: Abscess formation following a $1x10^6$ *iv* inoculum of *C. albicans*; ± less than one abscess per low power (x50) field (LPF), + 1-5 abscesses per LPF, ++ 6-10 abscesses per LPF, +++ more than 10 abscesses per LPF, NB abscesses were generally larger in CBA mice than in BALB/c mice and larger in primary infection than in secondary infection. Yeast load; ± occasional yeast/pseudohyphal cells in tissue parenchyma, + low yeast load per abscess, ++ moderate yeast load per abscess, +++ heavy yeast load per abscess. Immunohistology, ± occasional cell, + few cells present in abscesses, ++ moderate positive cell infiltrate in abscesses, +++ heavy infiltration of positive cells into abscesses.

PRESENTATIONS

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