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Antigen Processing and Presentation of *Cryptococcus neoformans*

by

Rachel Mona Syme

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ABSTRACT

Cryptococcus neoformans is an encapsulated yeast that is one of the most common invasive and fatal fungal infections in AIDS. Studies were performed to determine the contribution of T cell subsets to lymphocyte proliferation in response to *C. neoformans*. It was demonstrated that human CD4 and CD8 cells are activated, and proliferate in response to *C. neoformans*, however proliferation of CD8 cells was dependent upon CD4 cells. CD8 cells became activated in the absence of CD4 cells and expressed early, but not late components of the cell cycle. CD8 enriched cells failed to express mRNA for IL-2, suggesting that CD4-dependent IL-2 production was required for CD8 cell proliferation. However, IL-2 was not sufficient to restore CD8 cell proliferation. These studies support the clinical impression that CD4 cells are important in cryptococcosis, and suggest that appropriate CD4-derived signals could allow CD8 cells to assist in host defense.

The nature of antigen processing and presentation of *C. neoformans* was investigated by identifying the antigen presenting cell (APC). Dendritic cells were the most potent APC for *C. neoformans*. These cells ingested the organism and induced T cell responses. Use of the mannose receptor was found to be important in generating this response. To elicit T cell responses *C. neoformans* required extensive lysosomal processing by serine and aspartic proteases.

The effect of serotype and polysaccharide capsule on lymphocyte proliferation was examined. Lymphocytes responded to organisms of serotype A and D. Lymphocyte proliferation was greater following stimulation by minimally encapsulated strains than heavily encapsulated strains. A heavily encapsulated strain, did not suppress the response to an acapsular mutant however, the response to an acapsular strain was suppressed by addition of purified polysaccharide. Although cryptococcal polysaccharide (CPS) induces release of IL-10, it was not responsible for polysaccharide induced suppression. CPS also causes shedding of cell surface receptors, but did not diminish proliferation by affecting accessory molecules. Presence of CPS impaired phagocytosis, and this was critical for lymphocyte proliferation. CPS induced suppression could be overcome by opsonization with anticapsular antibody or human serum.

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DEDICATION

To Rob, who always wanted to be Dr. and Mr. Rachel Syme.

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ABBREVIATIONS

³ HTdR	thymidine
ADH	firmly adherent cells
AET	2-aminoethylisothiuronium bromide
AM	alveolar macrophage
APC	antigen presenting cell
BAL	bronchoalveolar lavage
CHCl ₃ :IAA	chloroform:isoamylalcohol
Con A	concanavalin A
cpm	counts per minute
CPS	cryptococcal capsular polysaccharide
CR	complement receptor
Crypto	<i>C. neoformans</i>
DC	dendritic cell
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAM	goat-anti-mouse
GAPDH	glyceryl aldehyde phosphate dehydrogenase
G-MCSF	granulocyte-macrophage colony stimulating factor
GXM	glucoronoxylmannan
HBSS	Hank's buffered salt solution
HEL	hen egg lysozyme
IL	interleukin
IL-2R α	interleukin-2 receptor alpha
L	loosely adherent cells
LAMP-1	lysosome associated membrane protein 1
Lf	leaf
LL	leucine methyl ester treated loosely adherent cells
LME	leucine methyl ester
LPS	lipopolysaccharide
mAb	monoclonal antibody
M-CSF	macrophage colony stimulating factor
MHC	major histocompatibility complex
MIIC	major histocompatibility class II compartment
MR	mannose receptor
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PE	phycoerythrin
SEB	Staphylococcal enterotoxin B
sem	standard error of the mean
SRBC	Sheep red blood cells
T	T cells
TCR	T cell receptor
tet	Tetanus toxoid
TNF	tumor necrosis factor

CHAPTER 1

INTRODUCTION

1.1 *Cryptococcus neoformans*

History/Characteristics

Cryptococcus neoformans is an encapsulated yeast, that can cause life threatening infections in immunocompromised individuals. It was first isolated in 1894 from peach juice by Sanfelice in Italy (Sanfelice, 1894), and the following year from a tibial lesion by Busse and Buschke in Germany (Buschke, 1895; Busse, 1895).

Cryptococcus neoformans is a member of the basidiomycotina. A large round or oval yeast, it varies in size from 4-12 μm (Levitz, 1991a). Two varieties of *C. neoformans* were distinguished in 1970 based on morphologic grounds; *C. neoformans* var. *gatti* and *C. neoformans* var. *neoformans* (Vanbreuseghem and Takashio, 1970). Subsequently, genetic, biochemical, serologic, ecologic and epidemiologic differences have been identified. *C. neoformans* var. *neoformans* is found worldwide, and has been isolated from soil and bird droppings (Levitz, 1991a). *C. neoformans* var. *gatti* is found in tropical regions of the globe, where the *Eucalyptus camaldulensis* tree is its ecological niche (Ellis and Pfeiffer, 1990a).

The organism possesses a number of important virulence factors including the ability to grow at 37°C, melanin, mating type, and polysaccharide capsule. The major

virulence factor for *C. neoformans*; however, is the capsule, which is synthesized after entry into the host (Farhi *et al.*, 1970). The primary constituent of polysaccharide is glucuronoxylmannan (GXM), which consists of a 1,3 mannose backbone with xylosyl and glucuronosyl side chains (Bhattacharjee *et al.*, 1984). The polysaccharide occurs in four serotypes. These serotypes differ in the extent of xylosyl substitution, and are all equally capable of inhibiting phagocytosis of the yeast (Kozel *et al.*, 1988a). Serotypes A and D are associated with *C. neoformans var. neoformans*, while serotype B and C are associated with *C. neoformans var. gatti*.

Serotype of the organism also appears to be an important component of virulence. Most cryptococcal infections are caused by *C. neoformans var. neoformans*, serotype A. In fact, in AIDS patients, infections are almost exclusively attributed to this serotype (Mitchell and Perfect, 1995; Dromer *et al.*, 1996b). This association is not due solely to the environmental prevalence of a particular serotype, as individuals greater than 60 years of age, of European descent, or who had received corticosteroid therapy are more susceptible to serotype D infections (Dromer *et al.*, 1996b). Further, the association of a particular serotype with disseminated forms of cryptococcosis has been reported among the immunocompromised (Cherniak and Sundstrom, 1994). This correlation between virulence and serotype has been proposed to be related to the chemical structure of the major component of the polysaccharide, GXM (Cherniak and Sundstrom, 1994).

How Acquired/Disease

Usually *C. neoformans* infects via the respiratory route. Basidiospores, of the sexual state of *C. neoformans* (Cohen *et al.*, 1982), as well as desiccated yeast cells (Ellis and Pfeiffer, 1990b) have been hypothesized to be the infectious form of the organism. Whether an infection is established or not then depends on the host's initial defenses, the size of the inoculum, and the virulence of the organism (Mitchell and Perfect, 1995). Most infections with *Cryptococcus* occur in the lung and central nervous system, although the involvement of many other organ systems has been described (Eng *et al.*, 1986). From the lung, *C. neoformans* can disseminate to cause life threatening systemic cryptococcosis, and to the central nervous system to cause cryptococcal meningitis. Common symptoms of cryptococcal infections include fatigue, headache, fever, and nausea (Chuck and Sande, 1989).

As this yeast rarely infects normal hosts, the risk for cryptococcal infections in the general population is low (about 0.15%/year) (Eng *et al.*, 1986). With the advent of immunosuppressive therapies in the 1960's cryptococcal infections became more common (Diamond, 1989). Today, patients who have underlying immunodeficiencies, or have impaired cell mediated immunity; such as individuals with organ transplants, chronic leukemias, lymphomas, HIV infection, or on corticosteroid treatments, are often hosts for cryptococcal infections (Diamond, 1989). Such associations implicate the importance of cell mediated immunity in combating this infection.

Cryptococcus and AIDS

C. neoformans is especially common in AIDS (Lewis and Rabinovich, 1972; Dismukes, 1988). It is the leading fatal fungal pathogen in AIDS (Perfect *et al.*, 1987; Dismukes, 1988; Coker, 1992) with the possible exception of *Pneumocystis carinii* (Armstrong, 1988; Murray and Mills, 1991), and the fourth most common pathogen overall. AIDS patients that fail to mount an effective immune response to this encapsulated yeast can develop life threatening manifestations of cryptococcosis including pneumonia and meningitis (Kovacs *et al.*, 1985; Eng *et al.*, 1986; Zuger *et al.*, 1986; Chuck and Sande, 1989). Once infected with *Cryptococcus*, 50% of AIDS patients get disseminated disease to organs apart from the meninges, such as the liver and kidney (Eng *et al.*, 1986). This dissemination is much lower in non-AIDS patients.

Recently, it was reported that up to 86% of cryptococcal infections in France were associated with AIDS (Dromer *et al.*, 1996a). Data from across the globe reports that 6-10% of patients with AIDS will develop cryptococcal meningitis (in nearly half of these patients it is also the AIDS defining illness) (Kovacs *et al.*, 1985; Eng *et al.*, 1986; Zuger *et al.*, 1986; Dismukes, 1988; Chuck and Sande, 1989; Seaton *et al.*, 1996). Prevalence rates range from 2-10% in Western Europe (Knight *et al.*, 1993), the United States (Kovacs *et al.*, 1985; Dismukes, 1988), and parts of South America (Bava and Negroni, 1992), to a high of 15-30% in some countries in Africa (Clumeck *et al.*, 1989). Prevalence rates in AIDS patients in South East Asia are quite variable. In one report from Thailand, 81% of AIDS patients at one hospital were suffering from cryptococcosis (Imwidthaya, 1994). More recently, it was reported that 15% of pulmonary infections in

AIDS patients, in Thailand, were attributed to *Cryptococcus* (Mootsikapun *et al.*, 1996); while in Papua New Guinea an infection rate of 9% has been documented (Seaton *et al.*, 1996).

Treatment

Once established, *C. neoformans* is difficult to eradicate. Until 1957, cryptococcal meningitis was nearly always fatal (Lewis and Rabionovich, 1972). Use of amphotericin B to combat cryptococcal infections has improved mortality rates (Spickard *et al.*, 1963; Sarosi *et al.*, 1969). At present, standard treatment for AIDS patients with cryptococcal infections includes a regime of amphotericin B and flucytosine (Bennett *et al.*, 1979; Sugar *et al.*, 1990) plus lifelong suppressive anti-fungal therapy with fluconazole (Bozzette *et al.*, 1991). This therapy, however, is still not adequate for AIDS patients (Sugar and Saunders, 1988). Despite receiving the best antifungal therapy available less than 50% of AIDS patients with a cryptococcal infections respond to treatment (Stevens, 1991). During initial therapy, mortality due to cryptococcosis is 10-25%, with a 12 month survival rate among patients of only 30-60% (Chuck and Sande, 1989). Most of this mortality can be attributed to devastating relapses. A more recent study reports a relapse rate of 40% (Dromer *et al.*, 1996a). Treatment becomes even more problematic with the emergence of amphotericin resistant (Powderly *et al.*, 1992), flucytosine resistant (Lyman and Walsh, 1992), and fluconazole resistant (Peetermans *et al.*, 1993) strains of *C. neoformans*.

1.2 Cell mediated immunity and *Cryptococcus*

The devastation of cryptococcal infections, especially in AIDS patients, has been the underlying force behind the increase in cryptococcal research over the past two decades. Apart from investigating different treatment regimes, research has focused on understanding the nature of the infection, the role of virulence factors, and how the precise nature of the immune response, and its components, play a role in host defense. The high incidence of cryptococcal infection in AIDS patients, suggests that cell mediated immunity is important in the clearance of *C. neoformans*. As well, the majority of infected non-AIDS patients also have cell mediated immune defects (Diamond, 1989). Work in murine models has confirmed this observation. Athymic nude mice have increased susceptibility to infections compared to their heterozygous litter mates (Graybill and Drutz, 1978). These mice can be protected by thymic transplantation or transfer of spleen cells from mice who have survived cryptococcosis (Graybill and Mitchell, 1979). Immunity to infection can also be accomplished by splenic T cell transfer from sensitized mice to non-immune mice (Lim and Murphy, 1980). Huffnagle and colleagues (1991a) found that athymic nude mice and SCID mice were both unable to clear infections with encapsulated strains of *C. neoformans*. These susceptible mice could be rendered resistant to cryptococcal infections by an infusion of T cells from immunocompetent donors, including immune T cells from the lungs. Further, mice depleted of their T cells are more susceptible to cryptococcal infections than control mice (Hill and Harmsen,

1991; Hill and Dunn, 1993). All these studies suggest that T lymphocyte responses are important in murine models of cryptococcosis.

Data from human studies is much more limited. Most information on the role of cell mediated immunity in combating cryptococcal infections in humans has been extrapolated from data on immunocompromised patients. Individuals with active cryptococcal infections have been shown to have depressed cell mediated immunity (Graybill and Alford, 1974), and patients with a variety of T cell immunodeficiencies are at an increased risk for cryptococcal infections (Mitchell and Perfect, 1995).

Cryptococcal infections are usually associated with CD4 counts of $<100 \text{ cells/mm}^3$ (Crowe *et al.*, 1991). These observations stress the necessity of intact cell mediated immunity to combat infection. If cell mediated immunity is intact, the organisms either fail to produce clinical symptoms, or do not disseminate from the lung (Lipscomb, 1989).

A model of *in vitro* responses where human peripheral blood mononuclear cells proliferate in response to the organism has been developed (Miller and Puck, 1984; Mody and Syme, 1993; Mody *et al.*, 1996) which allows for better investigation in humans. Using this model Hoy and colleagues (1988) have observed that PBMC from HIV infected patients failed to proliferate in response to *C. neoformans*, which correlated with progression to AIDS. Thus, when immunocompromised individuals are exposed to *C. neoformans* a cell mediated immune response that results in the activation and generation of effector cells fails to develop, which results in growth and dissemination of the fungi. These findings all suggest the importance of cell mediated immunity in combating *C. neoformans* infections.

T Cell Subsets

Knowing the importance of T cells in protection from cryptococcal infection, investigators have examined the nature of the T cell response by investigating the role of T cell subsets. Many researchers have looked at the role of CD4 and CD8 cells in murine models. Although AIDS patients have depressed CD4 cell function, most of the direct evidence for participation of CD4 cells in the immune response to *C. neoformans* has come from murine models (Mody *et al.*, 1990; Huffnagle *et al.*, 1991b; Hill and Harmsen 1991). Mice depleted of CD4 or CD8 cells are more susceptible to cryptococcal infections than undepleted control mice (Mody *et al.*, 1990; Mody *et al.*, 1993; Mody *et al.*, 1994). Further to this, Hill (1992) has shown that SCID mice reconstituted with CD8 cells are less resistant to cryptococcal infections than mice reconstituted with CD4 cells, however, both do better than untreated mice. Both CD4 and CD8 cells have been shown to proliferate in response to *C. neoformans*, in a murine model (Hill and Harmsen, 1991, Mody *et al.*, 1993; Mody *et al.*, 1994). CD8 cells accumulate in the lungs of CD4 deficient mice (Hill and Harmsen, 1991), suggesting these cells have a role in resistance to cryptococcal infections acquired by the lung. CD8 cells can also be required for delayed type hypersensitivity (Mody *et al.*, 1994). Clearance of *C. neoformans* from the lung corresponds with an influx of CD4 and CD8 cells (Huffnagle *et al.*, 1991b). Murine CD4 cells are essential for optimal clearance of *C. neoformans*, and murine CD4 cells proliferate following challenge with *C. neoformans* (Hill and Harmsen 1991; Huffnagle *et al.*, 1991b; Mody *et al.*, 1991).

Much less is known about the role of CD4 and CD8 cells in the human immune response to *C. neoformans*, and much of this is extrapolated from data from AIDS patients. The loss of CD4 cells correlates with increased susceptibility to cryptococcal infections in AIDS patients (Crowe *et al.*, 1991). However, there are no such diseases that produce an isolated loss of CD8 cells. An *in vitro* model of the anti-cryptococcal, cell mediated immune response has been proposed in which lymphocytes from healthy adults proliferate when cultured with *C. neoformans* (Graybill and Alford, 1974; Schimpf and Bennett, 1975; Miller and Puck, 1984). Lymphocytes from HIV-infected patients do not proliferate (Hoy *et al.*, 1988) suggesting that human CD4 lymphocytes are important in the proliferative response. However, Miller and Puck (1984) proposed that CD8 cells, but not CD4 cells proliferate in response to *C. neoformans*, as the proportion of CD8 cells was greater than CD4 cells after a period in culture. This is surprising based on murine studies and clinical data from AIDS patients that suggest an important role for CD4 cells in host defense to *C. neoformans*. Many questions remain unanswered about the human immune response to *C. neoformans*. It is not known whether CD4 or CD8 cells proliferate in response to *C. neoformans*, whether CD4 cells are required for activation and proliferation of CD8 cells, and what the nature of this putative CD4 signal would be. Murine studies suggest that the immune response to *C. neoformans* does not simply rely on a CD8 cytolytic or CD4 helper response. Therefore, it is essential to investigate the initiation of the immune response and the cells involved to being to comprehend how these responses are developed and determine the nature of the T cell response.

1.3 Outcome of Cell Mediated Immunity

Protective cell mediated responses can be carried out by a variety of effector cells. T cells can activate or recruit effector cells, or have cytotoxic abilities themselves. More specifically, T cells have the capacity to secrete cytokines that empower other cells of the immune system such as neutrophils, monocytes and natural killer cells. CD4 cells can recruit effector cells such as macrophages and granulocytes (Huffnagle *et al.*, 1994) by the release of cytokines, such as IL-5 (Huffnagle *et al.*, 1998). Cytokines, such as IFN- γ and IL-2, can activate effector cells for anticryptococcal activity (Mody *et al.*, 1991; Levitz and Dupont, 1993; Murphy, 1993). In fact, once a yeast is engulfed it is not killed unless the macrophage has been activated (Monga, 1981). CD8 cells may also participate by secreting activating cytokines (Gajewski and Fitch, 1990; Maraskovsky *et al.*, 1992), such as IFN- γ and granulocyte-macrophage colony stimulating factor (GM-CSF) which have been demonstrated to enhance anti-cryptococcal activity of effector cells (Flesch *et al.*, 1989; Mody *et al.*, 1991; Chen *et al.*, 1994; Kawakami *et al.*, 1995).

Another T cell cytokine, IL-10, has been shown to downregulate cell mediated responses such as T lymphocyte proliferation in response to *C. neoformans* (Monari *et al.*, 1997). IL-10 can also regulate the production of TNF- α produced by cells stimulated with *C. neoformans* (Levitz *et al.*, 1996). TNF- α , an important mediator of inflammation, is released by *C. neoformans* stimulated leukocytes (Levitz *et al.*, 1994a). It is critical for the development of protective T cell responses and promotes recruitment of immune cells (Aguirre *et al.*, 1995; Huffnagle *et al.*, 1996). Activated T cells can also secrete cytokines that enable other immune T cells to directly bind to and inhibit growth

of *C. neoformans* (Levitz, 1991b; Levitz and Dupont, 1993; Murphy, 1993; Levitz, 1994; Levitz *et al.*, 1994b). For instance, IL-2 activates CD4 and CD8 cells for cryptococcal killing (Levitz and Dupont, 1993).

Apart from cytokine production and activating effectors T cells can also kill infected cells. CD8 cells have been hypothesized to lyse *Cryptococcus* laden inactivated phagocytes (Huffnagle *et al.*, 1991b). T cells may also participate in host defense by sequestering infection. CD4 cells also have a role in containing cryptococcal infections. They enable multi-nucleated giant cells to form around and sequester *C. neoformans* at the primary site of infection (Hill, 1992). The cytokines most likely involved in these interactions include IFN- γ , IL-4, MIP-1 α and TNF- α (Levitz *et al.*, 1994a; Huffnagle *et al.*, 1996; Huffnagle *et al.*, 1997).

Cell mediated immunity is also important in the stimulation of protective antibody production. CD4 cells can secrete cytokines such as IL-4 and IL-5 that stimulate B cells. In CD4 depleted mice, IgG₁ mAb are not protective and IgG₃ mAb accelerate infection. In mice lacking CD8 cells, both IgG₁ and IgG₃ prolong survival, an effect mediated through IFN- γ . Thus, T cells are required for antibody mediated protection as there is functional dependence of passive antibody on T cell function (Yuan *et al.*, 1997).

1.4 Antigen Processing and Presentation

As most research has focused on the effector functions generated by a cell mediated immune response, little has been learned about how these protective responses

are generated. T cells responses can be generated by recall antigens, mitogens or superantigens. Much work from murine and human studies suggests *C. neoformans* is behaving as a recall antigen. Lymphocytes from uninfected mice do not proliferate in response to cryptococcal antigen (Mody *et al.*, 1993). This data is in agreement with earlier studies which demonstrated that this response was not mitogenic (Mody *et al.*, 1990). Further protection can be transferred to non-immune mice from those that have been sensitized (Graybill and Mitchell, 1979; Lim and Murphy, 1980). Miller and Puck (1984) developed an *in vitro* assay of cryptococcal responses. They found that an individual's lymphocytes proliferated in response to *C. neoformans* after 7-9 days in culture. If lymphocytes were given a secondary stimulation with the organism the response was both earlier and greater (typical of a recall response). The time course and magnitude of the responses of normal individuals was also different from cured patients, who had both earlier responses with a greater degree of stimulation (Miller and Puck, 1984). In another study, individuals who had recovered from cryptococcosis also had amnestic responses (Hoy *et al.*, 1989). In general, the magnitude and time course of lymphocyte proliferation to *C. neoformans* is typical of antigenic responses. Murine studies also demonstrate that generation of antigen-specific T lymphocyte responses to *Cryptococcus* requires processing, again suggestive of an antigenic response (Collins and Bancroft, 1991).

Antigen processing and presentation is the initiation point for the development of protective T cell responses, such as to *C. neoformans*, and is the major influence on the nature of the T cell response. The processing and presentation pathway begins with the

binding of organism to the surface of an antigen presenting cell (APC), uptake, degradation, association with a major histocompatibility complex (MHC) molecule and transport to the cell surface, expression on the cell surface, and finally recognition by the T-lymphocytes. This process will be discussed in detail, along with how the outcome of the T cell response might be influenced by this event.

Binding and Uptake

The first step in antigen processing and presentation involves binding and uptake. Capture can occur by binding of antigen to specific receptors, or by fluid phase endocytosis. The nature of the receptors on an antigen presenting cell in part determines whether the antigen/pathogen can bind to that cell. Receptors come in two varieties, antigen specific receptors such as membrane immunoglobulin, or those with specificity for components of complement or mannose/fucose.

Microbial attachment to a phagocyte is a prerequisite for engulfment. After binding of a particle to a phagocytic receptor, internalization of the particle may or may not take place depending on the receptors involved and the state of activation of the cell (Bianco *et al.*, 1975; Newman *et al.*, 1984). There are three general mechanisms for taking up large particulates, such as *C. neoformans*; receptor mediated endocytosis, phagocytosis and macropinocytosis. Endocytosis is a method of taking substances into a cell from the extracellular fluid through plasma membrane vesicles (Brown, 1995). Extracellular ligands bind receptors, such as FcR, which then concentrate in clathrin coated pits. The coated pits invaginate and bud off forming uniform vesicles of <200 nm

in size. Vesicles can then fuse to form endosomes. Endosomes can be classified either as early sorting endosomes or late endosomes. These two classes of vesicles vary in subcellular localization, morphology, physical characteristics and acidification properties (Gruenberg and Maxwell, 1995). Early endosomes are transferrin receptor (TfR) and rab4 positive, while late endosomes lose TfR, but express rab7, mannose 6- phosphate receptor and cathepsins (Gruenberg and Maxwell, 1995). Phagocytosis is used for the engulfment of microorganisms and tissue debris for specific and non-specific immunity (Brown, 1995). Antigen is recognized by receptors on the cell surface, which results in a signal to evoke cytoskeletal changes. These changes ultimately allow for uptake to occur. This dependence on cytoskeletal rearrangement is the major characteristic differing phagocytosis from receptor mediated endocytosis. Receptors involved in phagocytosis include FcR, complement receptors (CR) and mannose receptors (MR). Macropinocytosis is a form of non-selective endocytosis, which is not clathrin mediated (Swanson and Watts, 1995). The vesicles, formed by this folding back of the plasma membrane against itself, are heterogeneous in size (1-3 μm) and form at the leading edge of cells (Lanzavecchia, 1996). This unguided ruffling along the cell's surface eventually becomes a macropinosome. It is structurally similar to an actin rich pseudopod that makes a phagosome (Swanson and Baer, 1995).

The nature of this initial binding interaction is important in determining the amount of protein taken up, but also in establishing the type of the T cell epitopes generated. As the receptor employed may be recycled, or degraded along with the antigen, this would effect the peptides generated depending on which receptor was used

(Lanzavecchia, 1996). For instance, some receptors may protect certain antigenic sites from catabolism (Lanzavecchia, 1990). Ligands such as complement fragments or immunoglobulin that bind antigen before uptake have been demonstrated to modulate antigen processing (Rey-Millet *et al.*, 1994; Jacquier-Sarlin *et al.*, 1995). Covalent binding of C3b to tetanus toxoid both increased and prolonged stimulation of specific T cell proliferation as compared to tetanus toxoid alone (Jacquier-Sarlin *et al.*, 1995). These antigens also differed in their susceptibility to different proteases.

The mechanisms of uptake employed by a cell can also effect the endocytic compartment to which an antigen/pathogen is delivered. This would influence where processing takes place, and determine the presentation of different T cell epitopes (Amigorena and Bonnerot, 1998). Two compartments exist where peptides are generated and bind class II molecules. MHC class II compartments (MIIC) are compartments that contain newly synthesized MHC class II molecules, LAMP-1 (lysosome associated membrane protein 1) and cathepsin D (Castellino and Germain, 1995). These vesicles also contain HLA-DM, which functions as a peptide editor (van Ham *et al.*, 1996). During the maturation of dendritic cells MIIC convert to non-lysosomal vesicles that discharge MHC-peptide to the cell surface (Cella *et al.*, 1997). Early endosomes are the second compartment. These slightly acidic vesicles contain mature MHC II molecules that are recycled from the cell's surface (Reid and Watts, 1990). Some peptide MHC complexes are generated exclusively in the early endosome compartment (Pinet *et al.*, 1994) others in MIIC (Davidson and Reid, 1991). Peptides generated at an early stage along the endocytic pathway may bind recycled MHC II in

early endosomes, while peptides generated later along this pathway are loaded upon new MHC molecules (Zhong *et al.*, 1997).

The nature of the uptake mechanisms used, and the APC used to process *C. neoformans* may be a major determinant of the compartment the microbe is delivered to and the degree of proteolysis it undergoes. For instance, use of mannose receptors and macropinocytosis target antigens to MHC vesicles (Sallusto *et al.*, 1995). B cells have been shown to present pigeon cytochrome C to antigen specific T cells irrespective of the uptake mechanism employed, while macrophages could present the same antigen only when it was taken up via macropinocytosis (McCoy *et al.*, 1995). Specific receptors and uptake mechanisms of individual antigen presenting cell (APC) types will be discussed later along with each cell type.

Degradation and Association with MHC

After internalization, antigen is processed. There are two pathways of antigen processing; the endogenous pathway, and the exogenous pathway. The pathway that is engaged has enormous influence on the responding T cell population. The later pathway of antigen processing usually stimulates CD4 cells, which are primarily responsible for T cell help and delayed type hypersensitivity (DTH), while the former pathway usually stimulates CD8 cells, which are primarily cytotoxic or suppressor cells. These two pathways specifically determine the T cells that will respond including the cytokines released and the effector functions generated.

Antigens external to the cell become part of the exogenous processing pathway (Germain, 1994). Such antigens are phagocytized by the APC, and are subsequently processed by lysosomal enzymes in endocytic vesicles. The generated peptide fragments (15-23 aa) then bind to MHC class II molecules which are assembled in the endoplasmic reticulum from an alpha chain, a beta chain and the invariant chain. The invariant chain is felt to circumvent premature peptide binding to MHC (Elliott *et al.*, 1987), and to help transport the alpha- beta dimer from the trans-golgi into the endosomal compartment by acting as a chaperone (Lotteau *et al.*, 1991). Initially, within the endosome there is limited proteolytic digestion of the invariant chain which leaves an invariant chain peptide (CLIP; class II associated invariant chain peptide). CLIP is then exchanged for antigenic peptides, a function that is catalyzed by DM, an MHC encoded molecule (Denzin and Cresswell, 1995). Therefore MHC II molecules can only bind peptide in endosomes where the invariant chain is released and catabolized (Roche and Cresswell, 1990). The class II cleft is open ended allowing for the large variation in peptide size. The alpha 1 and beta 1 domains of MHC class II molecule constitute the peptide binding groove. After binding, this complex of MHC and peptide is transported to the cell surface for presentation to histocompatible CD4 positive T cells (Neefhes and Ploegh, 1992).

The endogenous pathway relies on endogenous antigens or antigen that gains access to the cytosol of the antigen presenting cell (Germain, 1994) often, but not exclusively due to translation. The cell's proteasome, a multicatalytic protease complex, degrades the endogenously synthesized antigen into peptides (Yang *et al.*, 1992). Low

molecular mass polypeptides 2 and 7 (LMP 2 and 7) are proteasome subunits. They are believed to have a role in cleavage, and bias the production of peptides towards those that are favored by MHC (Monaco, 1995). Cytoplasmic peptides are transported into the lumen of the endoplasmic reticulum by transporters associated with antigen presentation (TAPS) (Spies *et al.*, 1990; Kelly *et al.*, 1992). TAP molecules are composed of TAP1 and TAP 2. TAP1 and TAP2 are multimembrane spanning proteins essential for peptide loading of class I molecules. Together they form a heterodimer. TAP is specific for peptide length and peptide sequence. The generated peptides (8-11 amino acids (Van Bleck and Nathenson, 1990) then bind to nascent MHC I molecules and β 2-microglobulin, at the alpha 1 and 2 domains, in the endoplasmic reticulum. Binding of the peptides releases the complex from TAP. Mutant cells that lack TAP genes are unable to present most endogenous peptides to CD8 cells (Spies and DeMars, 1991). Class I molecules bind peptides of a restricted size because the amino and carboxy termini have to bind with the ends of the class I peptide to ensure a stable interaction (Fremont *et al.*, 1992). The stable complex of MHC I, β 2-microglobulin and peptide leave the endoplasmic reticulum and are transported through the Golgi and trans-golgi to finally be expressed on the cell surface and eventually presented to histocompatible CD8 positive T cells.

In part, because of its potential to gain access to the intracellular space and survive intracellularly, *C. neoformans* has the potential to stimulate either the exogenous or endogenous pathway (Mitchell and Friedman, 1972; Herrmann *et al.*, 1994; Lee *et al.*, 1995). *C. neoformans* can replicate intracellularly (Diamond and Bennett, 1973), and can

become extraphagosomal (Lee *et al.*, 1995). It therefore has the potential to be released into the intracellular space and gain access to the endogenous antigen processing pathway. Other organisms, such as live *M. bovis*, survive within host cells and release antigen into the endogenous antigen processing pathway for activation of CD8 T cell responses (Turner and Dockrell, 1996). It has not previously been determined whether *C. neoformans* antigens can be presented by MHC I to CD8 cells.

Although exogenous antigen is usually presented by class II MHC, exceptions do exist. Exogenous antigens have been shown to stimulate class I responses by transferring to the endogenous pathway (Kovacs-soncs-Bankowski and Rock, 1995; Norbury *et al.*, 1995; Reis e Sousa and Germain, 1995). In these instances, class I presentation was enhanced if soluble antigen was associated with latex beads or co-administered with the beads (Reis e Sousa and Germain, 1995; Kovacs-soncs-Bankowski and Rock, 1995). It is possible that the particulate nature of *C. neoformans* would mimic this effect. Class I presentation can also occur if membrane ruffling or macropinocytosis are stimulated by phorbol ester or macrophage colony stimulating factor (M-CSF) (Norbury *et al.*, 1995). Phagocytic overload has been proposed as another mechanism of exogenous antigen being presented by class I molecules. A loss of membrane integrity would result, and egress of target antigen into the cytosol could occur (Reis e Sousa and Germain, 1995). One could imagine this happening if antigen presenting cells were engulfing and degrading large microbes such as *C. neoformans*. This would suggest that class I presentation could occur and activation of CD8 cells would result. "Endogenous" antigens have also been described that gain access to the exogenous pathways, and

generate class II responses (Jaraquemada *et al.*, 1990; Dodi *et al.*, 1994). Higher doses of antigen (virus) and longer infection time can increase this probability (Jaraquemada *et al.*, 1990). This seems unlikely to be a major contributor of CD4 T cell responses to *C. neoformans*.

Over the last decade there has been a great deal learned about antigen processing pathways in general; however, very little has been learned about processing of fungal antigens. As fungi, such as *C. neoformans*, are becoming more significant pathogens, it is imperative to have a good understanding of how immune responses to these pathogens develop and the nature of the response that is generated.

1.5 Antigen Presenting Cells

The processing and presentation pathway, such as to *Cryptococcus*, begins with the antigen presenting cell. The cellular source of the APC can be integral in determining the outcome of a cellular immune response (Weaver *et al.*, 1988; Gajewski *et al.*, 1991). APC provide two functions in activating T cells. First, they convert antigen to peptides into a form that can be recognized by T cells. Secondly, they provide costimulatory activity. As previously discussed, the uptake mechanism that is utilized, the endocytic compartments that are utilized, and the proteolytic machinery that is available, all influence peptide generation. Activation of T cells by different APC, may also vary due to differences in their endocytic and processing properties including protease composition and compartments (Scherer *et al.*, 1989; Schneider and Sercarz, 1997). For instance, dendritic cells have been reported to have an abundance of MHC vesicles, B cells much

fewer and monocytes a very low number (Nijman *et al.*, 1995). The quality and quantity of epitopes generated by particulate and soluble antigens was found to depend upon whether B cells or macrophages were the APC, (Vidard *et al.*, 1996), suggesting there are differences in how these cells process and present antigen. Macrophages were found to be more efficient in presenting particulates and could present antigen from particles of a wider range of sizes. Such differences have also been demonstrated for B cells and dendritic cells (Gapin *et al.*, 1998).

Internalization by different routes can target antigen to different compartments (Fineschi and Miller, 1997). McCoy and colleagues (1993) demonstrated that B cells can present antigen targeted to an early compartment by the transferrin receptor, while a macrophage can only process this antigen if it is internalized by macropinocytosis. The cell type (origin) of the APC can also influence the nature of the T cell response by affecting the type and amount of costimulation. For example, different APC may provide different second signals such as cytokines and costimulatory molecules or cofactors (Gajewski *et al.*, 1991; Thompson, 1995). These signals include adhesion molecules like ICAM-1, ICAM-2, ICAM-3 and LFA-3, as well as the costimulatory molecules B7-1 and B7-2. Costimulatory molecules regulate growth and differentiation of T cells. Engagement of B7 by CD28 (on a T cell) enhances transcription of cytokine genes. Expression of B7-1 or B7-2 by an APC can affect the type of immune response developed (Kuchroo *et al.*, 1995; Lenschow *et al.*, 1995). In one system B7-2 expression was associated with the development of a Th2 cytokine pattern, while B7-1 was associated with a Th1 pattern (Thompson, 1995). Presentation by B cells favors Th2 responses

while presentation by macrophages favors Th1 responses (Paul and Seder, 1994). B cells result in optimal proliferation of Th2 cells, while adherent cells are best for Th1 cell proliferation (Gajewski *et al.*, 1991). These differences are speculated to be due to difference in cofactor expression by these two APC populations.

Many different types of cells can act as APC. “Professional” antigen presenting cells include macrophage/monocytes, B cells, and dendritic cells. These cell populations are considered “professional” because they all have efficient mechanisms of antigen uptake, have costimulatory capacity and a level of MHC class II expression along with the ability to efficiently generate a specific determinant/MHC complex when compared to other cell types. These three APC populations utilize different methods of antigen uptake, have different tissue distribution, as well as different uptake and processing capabilities. These will be discussed along with each cell type. All are possible candidates as the antigen presenting cells for *C. neoformans*.

Macrophages

Macrophages are APC found in most tissues. They are highly secretory cells which can release a myriad to cytokines. These multifaceted cells may function as immune effectors, as well as antigen presenting cells. Intracellular processing of antigen and its presentation to T cells has been demonstrated by macrophages for a variety of antigens and microbes (Unanue and Allen, 1987). Macrophages can take up antigen via phagocytosis or macropinocytosis, and are unique in their high degree of phagocytic capacity. They can engulf much larger particles than B cells, and have the ability to

engulf live pathogens via phagocytosis (Unanue, 1984). This capacity to take up microbes, such as *Listeria monocytogenes*, has been demonstrated to result in antigen presentation to T cells (Unanue and Allen, 1987).

Macrophages express a range of receptors to immunoglobulin, complement and glycoproteins (FcR, CR3, MR) which allow them to interact with a variety of ligands (Manca *et al.*, 1991; Stahl, 1992). All are important in giving these cells their broad phagocytic ability. These cells can provide costimulation, but have been reported to express potent costimulatory activity only when stimulated by microbes or other activators such as IFN- γ (Liu and Janeway, 1991). For instance, they increase their expression of MHC II after IFN- γ exposure (Takemura *et al.*, 1984) which increases their presenting capacity.

C. neoformans is normally acquired by the respiratory tract, therefore the lung is the site of initial inoculation and host defense. Alveolar macrophages are found at this initial site of cryptococcal infection. Consequently, this would be the first phagocytic cell that *C. neoformans* would come into contact. The primary function of alveolar macrophages is to remove unwanted particles from the lung (Green and Kass, 1963). These cells can inhibit replication of *Cryptococcus* (Weinberg *et al.*, 1987; Wagner *et al.*, 1992), and have been demonstrated to kill the organism when activated (Mody *et al.*, 1991). Their antigen presenting capacity is debatable. Previous studies have demonstrated that these cells are immunosuppressive for T cell responses (McCombs *et al.*, 1982; Toews *et al.*, 1984). As well, alveolar macrophages do not migrate to secondary lymphatic organs to successfully present antigen to T cells (Havenith *et al.*,

1993a, 1993b). However, these cells have the advantage of being highly phagocytic and could deal with a large and robust microbe like *Cryptococcus*. Further, they have been described taking up whole *C. neoformans* (Bulmer and Tacker, 1975), and have been reported to present unencapsulated organisms to T cells, but they are not as effective for encapsulated strains (Vecchiarelli *et al.*, 1994a; Vecchiarelli *et al.*, 1994b). Despite the relevance of alveolar macrophages in cryptococcal infections, their role as an APC in pulmonary resistance to infection is unclear.

Monocytes/macrophages are potential candidates for the APC for *C. neoformans*. They are highly phagocytic cells, and appear to have the necessary receptors and costimulatory function to process and present a large microbe like *C. neoformans*. A monocyte has always been assumed to be the major APC for *C. neoformans* (Collins and Bancroft, 1991), but the primary APC for presentation of *C. neoformans* has never been investigated.

B cells

B cells are also capable of presenting antigen to T cells (Chestnut and Grey, 1986) if activated (Krieger *et al.*, 1985). In particular, following clonal expansion, the antigen specificity of the surface immunoglobulin, which is used to capture antigen makes these cells extremely good APC for antigen specific recall responses (Chestnut and Gray, 1986). Antigen specific uptake by B cells is via the B cell receptor, membrane immunoglobulin (Bonnerot *et al.*, 1995), as the antibody involved in uptake can actually influence degradation of bound antigen. The receptor is degraded along with antigen and may act by protecting certain protease sensitive sites (Davidson and Watts, 1989).

Because of the expression of surface immunoglobulin these cells are also effective at presenting proteins that are present in low concentration (Constant *et al.*, 1995). These cells can also utilize other receptors to capture antigen including CR2, and FcR (Chestnut and Grey, 1986).

Resting B cells are CD80 negative, and a poor source of costimulation (Lasalle and Hafler, 1994). Activation, however, can induce expression of costimulatory molecules such as B7/BB-1 (Lasalle and Hafler, 1994). B cells also express low levels of MHC class II, which increases when the cells are activated (Metalay *et al.*, 1989). This upregulation improves their ability to function as antigen presenting cells. Thus, B cells can be effective APC as they express immunoglobulin to specific antigen, and constitutively express MHC II. However, to function effectively as APC they must first be activated. It is unlikely that B cells would be a good APC for *C. neoformans* as they usually do not internalize large particulate material (Unanue, 1984), compared to macrophages, which present larger antigen particles (Vidard *et al.*, 1996). Further, B cells have not been described as taking up and processing whole organisms, and lack expression of receptors such as the mannose/fucose receptor.

Dendritic Cells

Dendritic cells are potent APC that are integral in the initiation of T cell immunity. They are found in small numbers in lymphoid and nonlymphoid tissues, and account for less than 2% of blood leukocytes. They are bone marrow derived, but whether they are of lymphoid or myeloid lineage is still under debate (Inaba *et al.*, 1993;

Galy *et al.*, 1995). In general, dendritic cells are difficult to identify due to the lack of specific markers. They do, however, have a characteristic dendritic morphology, are potent stimulators of mixed lymphocyte reactions and can function at very low cell number compared with other APC. Dendritic cells are usually identified as different cell populations based on their location - the veiled cells of the afferent lymph, the Langerhans cells of the skin, the interdigitating cells of T cell dependent areas, and dendritic cells of peripheral blood (Lambrecht *et al.*, 1996). Dendritic cells from all tissues are potent APC for T cell dependent responses (Metaly *et al.*, 1989). These cells are unique among APC with regards to their ability to activate naive T cells. This ability gives dendritic cells a central role in the initiation of immune responses.

As dendritic cells mature, both their phenotype and function change. Progenitor or "immature" dendritic cells have increased endocytic capacity, lower MHC class II expression and reduced immunostimulatory ability (Scheicher *et al.*, 1995) as compared to their more mature counterparts (Sallusto and Lanzavecchia, 1994; Sallusto *et al.*, 1995). Immature dendritic cells have a rounded appearance; as the cell matures it expresses long processes (Ossevoort *et al.*, 1992; O'Doherty *et al.*, 1993). Pathogenic molecules like lipopolysaccharide (LPS) or cytokines such as TNF- α and GM-CSF will induce maturation of dendritic cells (Caux *et al.*, 1992; Sallusto and Lanzavecchia, 1994). Mature dendritic cells express high levels of costimulatory and adhesion molecules such as ICAM-1, LFA-3 and B7/BB1, making them even more immunostimulatory (Freudenthal and Steinman, 1990; Sallusto and Lanzavecchia, 1994).

Historically, studies have indicated that dendritic cells are not highly phagocytic (Steinman and Cohn, 1974; Inaba *et al.*, 1990). More recently, these cells have been shown to take up particulate antigen, such as lucifer yellow and rhodamine dextran by fluid phase endocytosis, as well as other APC populations (Levine and Chain, 1992). Subsequently, dendritic cells have been shown to be capable of macropinocytosis (Sallusto *et al.*, 1995); receptor mediated endocytosis (Reis e Sousa *et al.*, 1995), uptake of mannosylated glycoproteins via the mannose receptor (Engering *et al.*, 1997; Tan *et al.*, 1997), transfer of macrophage derived peptides (Havenith *et al.*, 1993a; Nair *et al.*, 1995) and FcR mediated phagocytosis (Sallusto and Lanzavecchia, 1994).

Dendritic cells have also been reported to have the capacity to internalize a variety of microbes including the *Bacillus Calmette-Guerin* (BCG) strain of mycobacteria. (Inaba *et al.*, 1993), *Listeria monocytogenes* (Guzman *et al.*, 1995), *Bordetella bronchiseptica* (Guzman *et al.*, 1994), *Escherichia coli*, *Salmonella typhimurium* (Svensson *et al.*, 1997) and *Mycobacterium tuberculosis* (Henderson *et al.*, 1997). Murine dendritic cells have been described as being able to take up various microbial pathogens, and then mediate antigen specific T cell responses (Moll *et al.*, 1995; Mbow *et al.*, 1997). More recently, human dendritic cells have been shown to take up the spirochete *Borrelia burgdorferi*, and induce potent T cell responses (Filgueira *et al.*, 1996). Dendritic cells, however, have not been shown to take up and present parasites or fungal pathogens.

Expression of the mannose receptor allows for recognition of mannans from bacteria and yeast by Langerhan's cells (Reis e Sousa *et al.*, 1993). In fact, an increase in the degree of glycosylation can increase uptake by the mannose receptor and is speculated

to lead to improved presentation (Lorenz *et al.*, 1990; Engering *et al.*, 1997). This could be a possible mechanism to take up highly mannosylated microbes such as *C. neoformans*.

Dendritic cells have a number of other characteristics that make them very potent APC. These cells are unique in their ability to cluster with one another and other cells directly during an immune response (Steinman, 1991). At the same time these cells lose their antigen capturing ability. This ability to capture antigen and then mature into a cell capable of stimulating T cells makes dendritic cells particularly potent APC. Upon activation, dendritic cells migrate to secondary lymphocyte organs to complete their maturation and present antigen to T cells. MHC antigen complexes remain stable on the cell surface for days (Pierre *et al.*, 1997; Cella *et al.*, 1997). Dendritic cells can present antigen in the context of MHC class II to CD4 cells or on MHC I to CD8 cells, and cause these cells to proliferate vigorously (Banchereau and Steinman, 1998). Dendritic cells constitutively express many costimulatory molecules (Steinman, 1991). High expression levels of costimulatory molecules like B7/BB1 give dendritic cells superior immunogenic potential to macrophages or resting B cells (Larsen *et al.*, 1992). Recently, it has been postulated that a CC-chemokine (DC-CK1) expressed by dendritic cells preferentially attracts CD45RA+ cells (Adema *et al.*, 1997). This would aid in dendritic cells ability to stimulate naive cells.

Although blood dendritic cells can activate Th1 and Th2 clones to a similar extent to monocytes and B cells, and memory and effector cells respond to all APC including resting B cells; dendritic cells can generally function at a much lower number. For

example, dendritic cells are 10-100 fold more potent than monocytes or B cells in generating a mixed lymphocyte response (Egner *et al.*, 1993; Roth and Speigelberg, 1996). These cells are believed to require much lower levels of antigen to induce T cell responses (Steinman and Swanson, 1995). Primary T cells can respond to much lower levels of antigen such as SEB if it is presented in context of a dendritic cells as compared with B cells and monocytes (Bhardwaj *et al.*, 1993). Dendritic cells and B cells use endocytosis primarily as a mechanism for presentation, while macrophages and monocytes also use endocytosis to carry out their functions as effector cells. Both dendritic cells and macrophages are capable of presenting some exogenous antigens in context of MHC I while B cells can not (Rock *et al.*, 1993), suggesting that if B cells were the main APC they would be unlikely to generate a CD8 cell response to *C. neoformans*. In general, B cells and macrophages are less efficient presenters than dendritic cells as dendritic cells constitutively express many costimulatory molecules. All three professional antigen presenting cell populations have the capability of stimulating T cells; dendritic cells, however, are felt by many to be the only cell type capable of stimulating a naive T cells. Although dendritic cells possess receptors that could interact with *C. neoformans*, and under some circumstances can be phagocytic, they have never been reported to ingest such a large microbe. Thus, it seems probable that monocytes/macrophages will be important antigen presenting cell for *C. neoformans*.

1.6 Antigen processing and presentation of *C. neoformans*

Understanding the nature of antigen processing and presentation, such as that required by *Cryptococcus*, begins with the antigen presenting cell. The APC is important in determining the outcome of the response as discussed above. APC are required in the lymphocyte proliferative response to *C. neoformans*; (Miller and Puck, 1984) however, the type of cell and the mechanism of antigen processing required for the development of T cell immunity has not been determined. That is the nature of the antigen presenting cell for *C. neoformans* within peripheral blood, or tissues has not been determined. It is believed that cells from bone marrow/in the blood, such as early dendritic cells, correspond to those cell found in peripheral tissues (Knight *et al.*, 1992; Filgueria *et al.*, 1996; Pierre *et al.*, 1997). Peripheral blood also represents the site of new APC, on their way to inflammatory sites. The APC could pick up antigen within the blood, when the pathogen has begun to spread hematogenously, or once within a tissue the APC could pick up antigen there. It is likely that most uptake occurs within tissues. APC in the blood can also consist of migrating APC that are now transporting antigen to secondary lymphoid organs (Larsen *et al.*, 1990; Austyn *et al.*, 1993).

Processing

After taking up an antigen/pathogen an APC must begin processing it. The nature of processing required to liberate an antigen can vary substantially between different types of antigen presenting cells (Schneider and Sercarz, 1997). *Cryptococcus* has a number of unique features that may require specialized processing and presentation,

including its large size and sturdy cell wall. The yeast is 4-12 μm in diameter (Levitz, 1991a) with a capsule ranging in size from 1-30 μm (Perfect, 1989). As previously discussed size may limit the APC type available for processing (Unanue, 1984), and the carbohydrate composition may inhibit processing (Gonzalez-Fernandez, 1997). The unique responses generated in response to *C. neoformans* also suggests that novel processing and presentation requirements may exist. For instance CD8 cells are important in delayed type hypersensitivity (DTH) responses to *C. neoformans* (Mody *et al.*, 1994) which is a function usually conferred by CD4 cells. As well, cryptococcal infections can generate an uncommon and complicated cascade of T suppressor cells (Murphy and Moorhead, 1982; Murphy and Mosley, 1985; Murphy *et al.*, 1988). Together these characteristics suggest a novel pathway of processing and presentation may be employed to generate these less common cell mediated immune responses, or the hypothesized various peptides generated from *C. neoformans* may be responsible for these different responses (Mody *et al.*, 1996).

Very little is known about the processing requirements of *C. neoformans*. Only one study by Collins and Bancroft (1991) has looked at the processing requirements of *C. neoformans* for T cell recognition. This study, in a murine model, found that within 1-2 hours after interaction of intact yeast cells with APC, the antigen was available for presentation. Processing of the antigen was inhibited in the presence of the lysosomotropic agent chloroquine. Their studies also suggested that presentation of *C. neoformans* is class II restricted in a murine system. Although these investigations provide some insight into antigen processing and presentation of *C. neoformans* they

leave many questions unanswered. The nature of the APC has been inferred to be a monocyte, but this has never been investigated. The nature of receptors involved in the generation of T cell responses and the processing requirements of *C. neoformans* are also unknown. As well, the specific proteases involved in generating effective responses are unknown.

1.7 Effects of virulence factors on antigen processing and presentation

The virulence of *C. neoformans* has a tremendous influence on host defense. Various characteristics of *C. neoformans* may influence the antigen processing pathway. The organism's ability to survive intracellularly may effect its processing and ultimately the outcome of the response like other fungi, such as *Histoplasma capsulatum* (Eissenburg *et al.*, 1993). Some organisms may alter processing by surviving in the phagolysosome of macrophages by preventing acidification (Eissenburg *et al.*, 1993). Other pathogens may survive by becoming extraphagosomal. Initially, an exogenous pathogen, such as *C. neoformans*, could easily be taken up and presented to CD4 cells. By becoming extraphagosomal the organism gains the potential to access the endogenous pathway, and allow for presentation to CD8 cells. Here the nature of the organism would be influencing the outcome of antigen processing and presentation.

The serotype may also be important in determining the nature of the immune response. For instance infections with serotype A organisms of *C. neoformans* are the most common (Bennett *et al.*, 1977), as has been discussed. This implies that serotype A organisms may have an advantage over serotype D organisms, such as being less

immunogenic. This difference has been speculated to be related to the chemical structure of GXM (Cherniak and Sundstrom, 1994).

Another major virulence factor for *C. neoformans* is its polysaccharide capsule. Cryptococcal polysaccharide (CPS) can suppress both humoral and cell mediated immune responses (Murphy and Cozad, 1972, Murphy and Moorhead, 1982; Murphy, 1988; Collins and Bancroft, 1991; Vecchiarelli *et al.*, 1994b). CPS can induce proinflammatory (Retini *et al.*, 1996) IL-6, IL-1, IL-8; and immunosuppressive cytokines (Vecchiarelli *et al.*, 1996) such as IL-10. CPS can also inhibit TNF- α secretion and IL-1 β production (Vecchiarelli *et al.*, 1995). It can also cause shedding of surface molecules (Dong and Murphy, 1996). Although the molecules identified by Dong and Murphy are not directly involved in antigen presentation, if others were shed, it could impair antigen presentation. CPS has been shown to induce T suppressor cells, which depress various T cell independent and dependent functions such as macrophage responses (Breen *et al.*, 1982; Blackstock *et al.*, 1987). It has also been demonstrated to suppress both specific and nonspecific antibody responses, which could effect induction of T cell responses (Murphy and Cozad, 1972; Kozel *et al.*, 1977; Breen *et al.*, 1982; Yuan *et al.*, 1997).

Capsule polysaccharide is antiphagocytic and can inhibit cryptococcal uptake by macrophages, monocytes and neutrophils (Kozel *et al.*, 1988a). Bulmer and Sans (1968) demonstrated that when an encapsulated strain of *C. neoformans* was cultured in the nonencapsulated state the rate of phagocytosis was three times greater than for the encapsulated form. Cryptococcal uptake is inversely proportional to capsule size

(Mitchell and Friedman, 1972). It is presumed that cryptococcal polysaccharide inhibits phagocytosis by blocking recognition of the yeast cell (Kozel, 1977).

The role of cryptococcal polysaccharide in the induction of human lymphocyte responses has only been superficially investigated. Encapsulation has been shown to diminish antigen specific T cell responses in murine models (Collins and Bancroft, 1991). Studies by Vecchiarelli and colleagues (1994b) suggest that encapsulation of *Cryptococcus* could down regulate the development of immune responses mediated by *Cryptococcus* laden alveolar macrophages by decreasing antigen presentation. Thus, there is the potential for CPS to suppress the development of T cell responses by inhibiting the uptake of *C. neoformans* by accessory cells, by inhibiting cell-cell interactions necessary for antigen presentation to T cells or by stimulating production of immunosuppressive cytokines. If CPS were to inhibit antigen presentation by inhibiting uptake of the organism, there is the potential to overcome this effect by opsonizing the organism, all possibilities to be investigated.

Summary paragraph

C. neoformans is a serious infection of AIDS patients. Research over the past decade has revealed many of its unusual properties, and much about humoral and cell mediated responses and effector functions. Little is know, however, about how the cell mediated response is generated and the effect that virulence factors of the organism have on cell mediated immunity. By systematically investigating this pathway of processing, presentation and T cell responses, the novel components of a response to *C. neoformans*

have been identified, and will hopefully aid in the development of immunotherapeutic strategies, vaccine development and our general understanding of immune responses to this important emerging fungal pathogen.

HYPOTHESES

C. neoformans will have unique processing and presentation requirements due to its structure, size, and virulence factors.

The organism will require uptake and processing by a phagocytic cell to generate T cell responses involving both CD4 and CD8 cells.

OBJECTIVES

1. To determine the role of human CD4 and CD8 cells in response to *C. neoformans*.
2. To determine the antigen presenting cell requirement of *C. neoformans*.
3. To determine the processing requirements for *C. neoformans*.
4. To determine the effect and mechanism of action of cryptococcal polysaccharide on cell mediated responses.

CHAPTER 2

MATERIALS AND METHODS

2.1 Preparation of *C. neoformans*

C. neoformans strain 67, strain 3501, strain 613, strain 52D and strain 68 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Strain C3D was a gift from J. Perfect, Duke University, Durham, North Carolina. Strain 145 was a gift of J. Domer Tulane University Medical Center, New Orleans, Louisiana. Strain 6 was a gift of G. Toews, University of Michigan Medical Center, Ann Arbor, Michigan. Characteristics of the organisms are noted in Table 1.

The organisms were maintained as previously described on Sabouraud's slants (Difco, Detroit, MI) and passaged to fresh slants monthly (Mody *et al.*, 1988). Periodically the unencapsulated organisms were stained with mucicarmine to ensure that the strain remained unencapsulated. The encapsulated strains were periodically examined by india ink. For all strains the organisms were killed by autoclaving at 121°C for 15 minutes and stored at 4°C for up to 3 months.

Table 1: Strains of *Cryptococcus neoformans var. neoformans*

STRAIN	ATCC NUMBER	SEROTYPE	CAPSULE SIZE	REFERENCE
67	52817	-	acapsular	Jacobson <i>et al.</i> , 1982
3501	34873	D	minimal	Kwon-Chung <i>et al.</i> , 1980
68	24064	A	light	Wilson <i>et al.</i> , 1968
613	36556	D	light	Kozel and Cazin, 1971
145	62070	A	moderate	Small <i>et al.</i> , 1986
H99/C3D	-	A	minimal	Granger <i>et al.</i> , 1985
6	62066	A	heavily	Small <i>et al.</i> , 1986
52D	24067	D	prominent	Wilson <i>et al.</i> , 1968

2.2 Polysaccharide and Cryptococcal Antibodies

Cryptococcal polysaccharide was a gift from T. Kozel, University of Nevada, Reno, Nevada. Capsular polysaccharide was obtained from strain 52 (serotype D) ATCC 24067 and from strain 68 (serotype A) ATCC 24064. The method of preparation is as described (Kozel *et al.*, 1984).

The cryptococcal antibody (mAb 471; Spriopulu *et al.*, 1989) was also a generous gift from T.R. Kozel. This particular anti-cryptococcal antibody is isotype IgG₁ and binds organisms of either serotypes A or D.

2.3 Polysaccharide coating and treatment of *C. neoformans*

In some experiments acapsular *C. neoformans* was incubated in purified polysaccharide (2 mg/ml) for 1 hour at 37°C. Unbound polysaccharide was removed by washing in 1X phosphate buffered saline (PBS). Polysaccharide coated *C. neoformans* were then used in proliferation and phagocytosis studies. For some experiments heat killed *C. neoformans* were incubated for 1 hour at 37°C in human AB serum (BioWhittaker, Walkersville, Maryland), heat inactivated serum (56°C for 60 minutes), or in the presence of a cryptococcal antibody (10 µg/ml). After antibody or human serum treatment, organisms were washed in 1XPBS and then used in proliferation or phagocytosis assays.

FITC labeling *Cryptococcus neoformans*

Heat-killed organisms were incubated with 0.5 mg/ml FITC isomer I (Sigma, Mississauga, Ontario) in 1XPBS for 10 minutes at room temperature (Chaka *et al.*, 1995).

The organisms were then washed three times and suspended in PBS at a density of 1×10^8 yeast/ml. Aliquots of the suspension were placed into sterile microcentrifuge tubes and stored at -70°C until required.

2.4 Isolation of peripheral blood mononuclear cells (PBMC)

Human peripheral blood was obtained by venipuncture from healthy adults. Blood was anticoagulated by adding 10 units/ml of heparin (Organon-Teknika-Cappel, Scarborough, ON). Peripheral blood mononuclear cells (PBMC) were purified by centrifugation ($800 \times g$ for 20 minutes) on a ficoll-hypaque density gradient (Lymphoprep, Labquip, Woodbridge, ON). PBMC were harvested and washed three times in Hanks' balanced salt solution (HBSS, Gibco, Burlington, ON). Cells were then counted and suspended in complete medium containing RPMI 1640 medium (Gibco), 5% heat inactivated pooled human AB serum (BioWhitaker, Walkersville, Maryland), 2 mM L-glutamine (Gibco), 100 units of penicillin/ml, 100 μg streptomycin/ml, 0.2 μg amphotericin B/ml (Gibco), 1 mM sodium pyruvate (Gibco) and 0.1 mM nonessential amino acids (Gibco).

2.5 T cell isolation

In some experiments T cells were purified from PBMC. PBMC were adhered to plastic P100 petri dishes (Corning Glass Works; Corning, New York) at 30×10^6 /plate. The plates were then incubated at 37°C , 5% CO_2 for 2 hours. After 2 hours nonadherent cells were harvested and counted. T cells were further purified by rosetting

to sheep red blood cells (SRBC; Cedarlane, Hornby, Ontario). Briefly, nonadherent cells were resuspended at 10×10^6 /ml in 20% human serum RPMI. 5% aminoethylisothiuronium bromide treated sheep red blood cells (AET-SRBC; recipe below) were added at 1 ml of sheep cells to 20×10^6 diluted nonadherent cells. This suspension was incubated in a 37°C water bath for 10 minutes. Cells were then spun at 900 rpm for 10 minutes. The pelleted cells were incubated overnight at 4°C . The next morning the pellet was gently resuspended and the rosette positive cells purified by centrifugation on a ficoll-hypaque density gradient at $1200 \times g$ for 20 minutes. The rosette negative cells were harvested. The remaining medium was then removed and the red cells lysed with NH_4Cl lysing buffer (pH 7.4) for 2 minutes on ice. The remaining rosette positive cells were washed 3 times in HBSS before being loaded on a preincubated nylon wool column (at 70×10^6 cells/ column). Prior to being used the column was packed with 0.6 g nylon wool (Robbins Scientific; Sunnyvale, CA) washed in HBSS and then incubated for 1 hour with RPMI medium at $37^\circ\text{C}/5\% \text{CO}_2$. Cells were incubated on the column for 1 hour at $37^\circ\text{C}/5\% \text{CO}_2$. The nonadherent cells (T cells) were eluted off the column with complete medium.

In some experiments, PBMC were depleted of T lymphocytes by rosetting cells to SRBC and retaining the rosette negative fraction. These cells were washed 3 times and then resuspended in complete medium and called T depleted. T enriched cells were cells that had only undergone SRBC rosetting.

2- aminoethylisothiuronium bromide (AET) Treatment of Sheep Red Blood Cells.

Sheep red blood cells were prepared as previously described (Saxon *et al.*, 1976). Briefly, 25 ml of sheep red blood cells in Alsever's (Cedarlane) were washed 5 times in 1 x phosphate buffered saline (PBS). Sheep cells were then suspended in 0.8 g/20 ml AET (pH 9.0) (Sigma). Cells were incubated in a 37°C water bath for 15 minutes, and then were washed an additional 5 times. Finally, the washed cells were suspended in 50 ml RPMI and stored at 4°C for up to two weeks. When required the cells were washed 3 times in 1X PBS. 1 ml of SRBC were added to 20 ml of a 20% human serum RPMI solution. This was equivalent to a 5% AET-SRBC solution.

2.6 Cellular depletions

In some experiments, PBMC were depleted of CD4 or CD8 cells using iron conjugated anti-CD4 or anti-CD8 antibodies (Dyna, Oslo, Norway) at a bead to target cell ratio of 4:1. Cells were incubated in the appropriate amount of washed antibody and rocked at 4°C for 40 minutes. The cells were separated by exposing them to a magnetic field that separated antibody-bound from unbound cells. The cells were washed 3 times in this manner in PBS (1% fetal bovine serum; FBS). Cells depleted of CD4 or CD8 cells contained less than 2.5% of the contaminating cell population as determined by immunofluorescent staining and flow cytometric analysis.

2.7 Antigen presenting cell (APC) isolation

Firmly Adherent cells

Firmly adherent cells were obtained by incubating PBMC on P100 plastic petri dishes at 37°C in 5% CO₂ in RPMI medium. After one hour the nonadherent cells were removed, and the adherent monolayer washed twice. The adherent cell population was then removed using a cell scraper (Starstedt; Montreal, PQ) and resuspended in complete media.

Loosely adherent cells

Loosely adherent cells were obtained by incubating PBMC on P100 plastic petri dishes for 2 hours at 37°C in 5% CO₂ in RPMI medium (Van Voorhis *et al.*, 1982). Nonadherent cells were gently harvested and the adherent cells resuspended in RPMI medium and 0.1% human serum overnight at 37°C in 5% CO₂. 18 hours later the loosely adherent cells were carefully harvested and resuspended in complete media.

LME treatment

In some experiments cytolytic cells were depleted by treating cells with leucine methyl ester (LME; Sigma). PBMC or dendritic cells were incubated at (20×10^6) for 40 minutes at room temperature in RPMI containing 5 mM LME (Theile and Lipsky, 1982). After the incubation cells were washed 3 times in HBSS and resuspended in complete media. Depletion of cytolytic cells (CD14+ and CD56+) was assessed by flow cytometry for success of treatment.

Dendritic cells

Loosely adherent cells were immunolabelled with iron conjugated antibodies against CD3, CD14, CD19 (Dynal, Upsala, Sweden) and/or anti- CD56 (Becton Dickinson) and goat-anti-mouse (GAM) -Dynabeads at a bead to target cell ratio of 4:1. Antibodies and cells were incubated by rocking at 4°C for 45 minutes. The cells were separated by exposing them to a magnetic field that separated antibody-bound from unbound cells. The cells were washed 3 times in this manner in PBS (1% FBS). To determine the success of the depletion, cells were immunolabelled with fluorescent antibodies or GAM-FITC and examined by flow cytometry.

In some experiments dendritic cells were isolated by the MACS system (Miltenyi Biotec, Gladbach, Germany). Briefly, loosely adherent cells were washed and placed in 80 µl of MACS buffer (1XPBS, 5 mM EDTA (Sigma), 0.5% FBS) per 10^7 cells. 20 µl/ 10^7 cells of MACS anti-CD3, CD14, CD19 and/or CD56 were added to the cells. Cells and antibody were incubated for 12 minutes at 4°C. Cells were then washed in MACS buffer and resuspended in 500 µl of buffer. Cells were placed on a pre-washed BS column and run through with 15 ml of ice cold buffer. Cells that did not bind to the column were depleted of the specified markers. Successful depletion was confirmed by flow cytometry.

Bronchoalveolar lavage cells

Bronchoalveolar lavage (BAL) samples were provided by Dr. Chris Mody (Department of Microbiology and Infectious Diseases, University of Calgary) and Dr. Steven Field (Department of Respiratory Medicine, University of Calgary). BAL was

performed on individuals who were stable patients that were undergoing BAL for minimal hemoptysis and had no bronchoscopic or radiologic abnormality. Samples were collected into saline. Following collection, cells were immediately placed on ice. Crude separation of cells from mucous was achieved by filtration through a 200 μm sterile nylon filter. Cells were then washed 3 times in ice-cold HBSS by centrifugation at 400 x g for 10 minutes at 4°C. Cells were finally resuspended in RPMI media and counted.

2.8 Lymphocyte proliferation assays

To determine whether *C. neoformans* stimulated lymphocyte proliferation, cells (2×10^5 cells/well) were cultured in 96 well round bottom tissue culture plates. Whole *C. neoformans* (2×10^5 cells/well) were added to the cultures. Cultures were incubated for 5, 7 or 9 days at 37°C, 5% CO₂. Thymidine (³H-TdR) incorporation was determined as a measure of DNA synthesis. 1 μCi ³H-TdR (ICN, Montreal, PQ) was added to each well 16 hours before the end of incubation. At the end of the incubation period, cells were harvested on glass filters (Brandel Inc., Gaithersburg, MD) and counts per minute (cpm) were determined in a liquid scintillation counter (Beckman; Mississauga, ON). ³H-TdR incorporation into cultures containing *C. neoformans* alone as routinely less than 300 cpm. In other experiments to ensure that cells were proliferating, PBMC were harvested from stimulated or unstimulated cultures after 2 hours, 5, 7, or 9 days. Cells were then counted using a hemacytometer.

Control Stimuli

In some experiments cells were stimulated with 2.5-10 µg/ml Concanavalin A (Con A, Sigma), 1:10 Leaf units tetanus toxoid (Connaught Laboratories, Mississauga, ON) or 1 µg /ml Staphylococcal enterotoxin B (SEB, Toxin Technologies). To determine mixed lymphocyte responses T cells (2×10^5 /well) were put in culture with irradiated PBMC (12.5×10^4 /well) or dendritic cells (12.5×10^4 /well) of a histoincompatible donor. Mixed lymphocyte responses were determined 5 days later by thymidine incorporation.

Ammonium chloride (NH_4Cl) treatment

In some experiments a lysosomotropic agent (NH_4Cl ; 0.5-1 mM) was added to cultures to inhibit lysosome function (Zeigler and Unanue, 1982). Lymphocyte proliferation was then determined as before.

Protease Inhibitors

To examine the role of proteases various protease inhibitors were added to cultures as per Puri and Factorovich (1988). Protease inhibitors used were the serine/cysteine protease inhibitors antipain and leupeptin (1-100 µg /ml; Sigma), and the aspartic protease inhibitor pepstatin A (1-100 µg /ml; Sigma). In other experiments microtubular polymerization was inhibited by the addition of cytochalasin B (1-100 µg /ml; Sigma) to cultures.

Lidocaine Treatment

In some experiments lidocaine (10-10000 µM; Baxter; Mississauga, ON) was added to the culture wells to inhibit phagocytosis (Das and Misra, 1994). This drug has

no effect on T and B cell proliferation to mitogens when used in cell culture (Berkeley *et al.*, 1994).

Blocking antibodies.

For blocking experiments, PBMC were purified and stimulated with *C. neoformans* in the presence of various monoclonal antibodies (mAb). To stimulate cultures 60 or 120 ng of anti-CD8, anti-CD4 (Becton-Dickinson; San Jose, CA), both or mouse IgG kappa (Sigma) which served as a negative control were added directly to cultures. To block receptors on the surface of dendritic cells, cells were incubated in the presence of mannan (3 µg /ml; Sigma), mannose (control sugar; Sigma), anti-mannose receptor antibody (25 ng/ml; Pharmingen), or control antibody (IgG₁; 25 ng/ml Becton Dickinson).

In other experiments cells were incubated in the presence of anti-IL-10 (10 µg /ml; ATCC) to block the effect of this suppressive cytokine, or isotype matched control antibody (ATCC). Hybridoma JESS-19F (rat IgG₂b) was obtained from the ATCC. The antibody was purified from ascites produced in pristane primed nude mice, by injecting 2×10^7 hybridoma cells intraperitoneally. The antibody was purified over a protein G column (Pierce, Rockford, IL). The ability to bind IL-10 was confirmed by ELISA.

For activation blocking experiments, PBMC were stimulated with *C. neoformans* in the presence of various concentrations of anti-CD8 (anti-leu-2a), anti -CD4 (anti-leu-3a), both, anti-DR, anti-DP, anti-DQ, (all Becton-Dickinson) anti-HLA-ABC (Cedarlane), or mouse IgG₂ kappa (Sigma), IgG₁ (Becton Dickinson) as controls.

2.9 RT-PCR

RNA Extraction

PBMC, or enriched populations of cells were stimulated with *C. neoformans* strain 67 or left unstimulated in 96 well plates for 0, 6, 24, 48, 72 or 96 hours. Cells were harvested and pooled from 48 wells. Total RNA was isolated using a modified guanidinium isothiocyanate method (Wong *et al.*, 1994). Briefly, harvested cells were lysed with 1.8 ml of denaturing solution (solution D; 50 ml guanidinium stock, 360 μ l 2-mercaptoethanol). Guanidinium stock consisted of 250 g guanidinium (Sigma) 298 ml H₂O, 13.2 ml 1M Na- citrate (pH 7.0), and 26.4 ml 10% sarcosyl (Sigma). RNA was then purified by the phenol chloroform method (Chomczynski and Sacchi, 1987). To the lysed cell suspension 0.1 ml 2M NaAC (pH 4.0), 1.0 ml phenol (Gibco, BRL), and 0.2 ml CHCl₃:IAA (24:1) were added. This mixture was set on ice for 15 minutes and then spun for 10 minutes at 10K. The aqueous phase was collected, transferred to a clean tube. An equal volume of isopropanol was added. After 1 hour at -20°C the solution was spun at 10K for 30 minutes. The pellet was then resuspended in 0.3 ml of solution D and 0.3 ml of isopropanol. After 1 hour at -20°C the tubes were spun for 10 minutes at 10 K 4°C. The resulting pellet was then washed in 70% ethanol before being dried at room temperature. The pellet was then suspended in 10 μ l of DEPC treated sterile water. RNA was then quantitated using a Genequant spectrophotometer (Pharmacia) and stored at -70°C until required.

Reverse Transcriptase Reaction

The reverse transcription reaction was performed in a 20 μ l volume containing 2 μ l of 10XPCR buffer (Pharmacia, Uppsala, Sweden), 1 μ g RNA, 1 mmole of each deoxynucleotide triphosphate (Pharmacia), 2 μ l reverse transcriptase primers (p(N6), Pharmacia, and 1 μ l RNA superscript (Gibco). Conditions were as follows: 10 minutes at 21 $^{\circ}$ C for preincubation, 50 minutes at 42 $^{\circ}$ C for the reverse transcriptase reaction and 5 minutes at 95 $^{\circ}$ C to terminate the reverse transcriptase reaction. (Temp-Tronic Thermal cycler, Thermolyne, Dubuque, Iowa). The resultant cDNA product was stored at -20 $^{\circ}$ C until required.

PCR Reaction

The PCR reaction was performed as previously described (Wong *et al.*, 1994). Briefly, the reaction was carried out in a 50 μ l volume containing approximately 2 μ l reverse transcriptase (RT) product, 5 μ l 10XPCR buffer, 20 μ M of each deoxynucleotide triphosphate, 20 pmol each 5' and 3' primer pair, 2 units Taq1 DNA polymerase (Gibco). Cycling conditions were 94 $^{\circ}$ C for 1 minute, 55 $^{\circ}$ C for 30 seconds and 72 $^{\circ}$ C for 1 minute. The amount of RT products electrophoresed was adjusted to give equal signal for the internal control mRNA (glycerl aldehyde phosphate dehydrogenase; GAPDH), and the number of cycles was adjusted to ensure that amplification was logarithmic for both target and control mRNA. For GAPDH, 23 cycles were used, for IL-2 32 cycles were used, for cyclin E 32 cycles were used, and for cyclin B1 27 cycles were used. The PCR products were electrophoresed through a 1.8% agarose gels containing 0.2 μ g/ml ethidium bromide and visualized by UV flourometry as described (Wong *et al.*, 1994).

Primers

Primers for cyclin B1, E, IL-2 and GAPDH were synthesized using sequences obtained from GenBank (Wong *et al.*, 1994).

Cyclin E	5' AGTTCTCGGCTCGCTCCAGGAAGA,
	3' TCTTGTVTCGCCATATAACCGGTCA,
IL-2	5' GAAGGCCACAGAACTGAAACATCT,
	3' TACAATGGTTGCTGTCTCATCAGC,
GAPDH	5' CGGAGTCAACGGATTTGGTCGTAT
	3' AGCCTTCTCCATGGTGGTGAAGAC
Cyclin B1	5'GGACTGAGGCCAAGAACAGCTCTT
	3'CAGCTCCATCTTCTGCATCCACAT

2.10 Electron microscopy

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was generously performed by Dr. F. Green (Department of Respiratory Medicine, University of Calgary). Briefly, dendritic cells were incubated with *C. neoformans* at an effector to target ratio of 1:20 at 37°C. Eighteen hours later the cells were fixed in Karnovsky's fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer). Slides were made and the fixed cells were then washed in buffer and dehydrated in a graded concentration of ethyl alcohol, and subjected to critical point drying with CO₂. Samples were then covered with gold/palladium and examined with a Hitachi S-450 scanning electron microscope.

Transmission electron microscopy

Cells were incubated as for scanning electron microscopy. Cells were then fixed in Karnovsky's fixative for 45 minutes and then washed twice in cacodylate buffer and post fixed in 1% osmium tetroxide for 30 minutes. Cells were washed with buffer and then dehydrated through a graded series of ethanol. The pellet was embedded in Spurr resin (JBS; Dorval, PQ). Ultrathin sections were cut with a diamond knife and counter stained with uranyl acetate and lead citrate. Sections (90 nm) were examined with a transmission electron microscope (Hitachi H7000) at an acceleration voltage of 75 kV. Grids were generously prepared by Leona Barclay (Department of Surgery, University of Calgary).

2.11 Flow cytometry

Phenotype of isolated cell populations

To investigate cellular activation PBMC, CD4 enriched or CD8 enriched cells were harvested from stimulated or unstimulated cultures after 2 hours, 4 hours, 5, 7 or 9 days. Phenotypic analysis was performed by labeling with antibodies directed against specific T cell surface receptors. The following antibodies were used for immunolabeling: anti-CD3-FITC, anti-CD4-FITC, anti-CD8-FITC, anti-CD4-PE, anti-CD8-PE, anti-CD25-FITC, anti-CD71-FITC, mouse IgG1-FITC, mouse IgG1-PE, anti-CD3-PerCP, anti-CD69-PE (all from Becton- Dickinson). To analyze CD69, gates were placed on CD3 positive cells and then the gated population was assessed for CD69 expression. To analyze CD25 and CD71 expression cells were gated on forward and 90° light scatter to include all lymphocytes. Flow cytometry was performed using a FacScan Flow cytometer

(Becton Dickinson) with an excitation frequency of 488 nm. At least 10 000 events per sample were evaluated.

In other experiments cell purity of isolated populations was assessed using anti-CD14-FITC, anti-CD19-FITC, anti-CD3-FITC, anti-CD56-PE, anti-HLA-DR-FITC, (all from Becton Dickinson) and anti-CD83-PE (Immunotech, Marseille, France).

2.12 Phagocytosis Assays

To determine the amount of *C. neoformans* taken up by adherent PBMC phagocytosis assays were employed as previously described (Kozel, 1977) with slight modifications. Briefly, PBMC were cultured on 24 well plastic tissue culture plates containing plastic 13 mm coverslips (Nunc, Naperville, Illinois) for 1 hour at 37 °C in complete media. After one hour the nonadherent cells were removed and *C. neoformans* (1×10^6 / well) were added to the adherent cells. At various time points (1 hour, 2 hours, 4 hours, or 18 hours) media and unphagocytosed organisms were removed by washing with PBS. Coverslips were air dried and then fixed in methanol. Coverslips were then stained with Giemsa (ICN) and examined by light microscopy for the number of adherent cells that had *C. neoformans* associated with them (% phagocytosis), as well as the number of ingested *C. neoformans* per cell (phagocytic index) were recorded. At least 200 cells were counted per monolayer. In other experiments, dendritic cells were put in culture with *C. neoformans* and at various times phagocytosis was investigated. Cells were considered internalized by focusing up and down on the cell and further if a vacuole could be seen surrounding the organisms.

Flow Cytometric Phagocytosis assays

Uptake of *C. neoformans* was also assessed using flow cytometry. The technique was used as per Chaka et al (1995), with some modifications. Briefly, FITC-labeled *C. neoformans* were put into culture with freshly isolated dendritic cells. Each reaction mixture contained 10^6 cells and 10^7 cryptococcal organisms yielding an effector to target ratio of 1:10. Eighteen hours later cells were examined for internalized *C. neoformans* by flow cytometry. Cells were gated on forward and 90° light scatter to include all the dendritic cells and exclude “free” *C. neoformans*. The organisms that were attached to the cells were discriminated from those that had been internalized by fluorescence quenching with 0.2 mg/ml trypan blue for 10 minutes. Only internalized organisms kept their FITC fluorescence after quenching. Cells were run on the flow cytometer both before and after trypan quenching to determine the amount of internalized organisms.

2.13 Statistics

Data are given as mean +/- standard error of the mean (SEM) for the indicated number of experiments. Each experiment was repeated with different donors on different days. (³H)-thymidine incorporation is expressed as the mean counts per minute +/- SEM quadruplicated wells. To analyze data statistically a one-way analysis of variance (ANOVA) or a student's t test for paired variables were performed (Statview 512+, Brainpower Inc., Calabasas, CA). For these tests $p < 0.05$ was considered significant.

CHAPTER 3

T CELL RESPONSES

3.1 BACKGROUND

To date most evidence for the role of T cell subsets in the immune response to *C. neoformans* has come from animal models (Mody *et al.*, 1990; Hill and Harmsen, 1991; Huffnagle *et al.*, 1991b). Murine studies have demonstrated that CD4 cells proliferate and are required for optimal clearance of the organism following challenge with *C. neoformans* (Mody *et al.*, 1990; Hill and Harmsen, 1991, Huffnagle *et al.*, 1991b). Additionally, it has been shown that murine CD8 cells also participate in the clearance of *C. neoformans* (Hill and Harmsen, 1991; Huffnagle *et al.*, 1991b; Mody *et al.*, 1993), and can be required for delayed type hypersensitivity (Mody *et al.*, 1994). Although the role of lymphocyte subsets in murine cell mediated immunity has been well established, participation of CD4 and CD8 subsets in the human cell mediated immune response to *C. neoformans* is much less well understood.

To assess human cell mediated immune responses to *C. neoformans* an *in vitro* model of the anti-cryptococcal cell mediated immune response has been developed in which lymphocytes from healthy adults proliferate when cultured with the organism (Graybill and Alford, 1974; Miller and Puck, 1984; Mody and Syme, 1993). As

lymphocytes from HIV infected patients do not proliferate (Hoy *et al.*, 1988) this suggests that human CD4 lymphocytes are important in the proliferative response. It has been proposed by Miller and Puck (1984); however, that CD8 cells, but not CD4 cells proliferate in response to *C. neoformans*. This is surprising based on murine studies and clinical data from AIDS patients. The following studies were performed to determine whether human CD4 cells proliferate in response to *C. neoformans*, and whether they are required for the CD8 cell response.

Prior to examining lymphocyte subsets experiments were performed to ensure that CD3+ cells were proliferating in responses to the organism, and that T cells were the only population contributing to proliferation. To examine the response of lymphocyte subsets to *C. neoformans*, the increase in the number of CD4 and CD8 lymphocytes was studied after stimulation of unseparated T cells. The contribution of CD4 and CD8 subsets to lymphocyte proliferation was investigated by blocking activation of each subset. The absolute requirement for CD4 cells and CD8 cells was established by depleting peripheral blood mononuclear cells (PBMC) of CD4 or CD8 positive cells. The time course of activation of CD4 and CD8 cells in response to *C. neoformans* was determined by analysis of the expression of the early T cell activation marker CD69 and the T cell growth factor receptors CD25 (interleukin-2 receptor alpha; IL-2R α) and CD71 (transferrin receptor). CD69 is a C-type lectin that is expressed within hours of lymphocyte activation (Biselli *et al.*, 1992). CD25 and CD71 indicate cellular activation (Reed *et al.*, 1986; Waldman, 1991) and their expression is required for cells to proceed through the cell cycle (Reed and Nowell, 1988).

These preliminary experiments provide evidence that proliferation of CD8 cells requires the presence of CD4 cells. Subsequent experiments were performed to determine whether CD4 cells were necessary for activation of CD8 cells by *C. neoformans*, or whether CD4 cells provided a signal at some stage later in the cell cycle. To establish the independent role of lymphocyte subsets in the proliferative response to *C. neoformans* CD4 and CD8 enriched cells were stimulated with *C. neoformans*. The ability of CD8 cells to be activated independently of CD4 cells was determined by analyzing the early inducible T cell activation marker CD69.

In order to proliferate, lymphocytes must then progress through G1 and S phase of the cell cycle, which is controlled by activity of cyclin dependent kinases, which are in turn controlled by the expression of regulatory subunits call cyclins. Cyclin E is required for progression from G1 to S phase of the cell cycle (Koff *et al.*, 1993; Ohtsubo and Roberts, 1993), and is first expressed in mid-G1 prior to the restriction point of the cell cycle. Cyclin B1 is expressed later in the cell cycle and appears during S phase. Its associated kinase activity is much later than cyclin E at the G2 to M transition (Pines and Hunter, 1992). Cyclin B1 controls mitotic entry and exit, and is required if a cell is to go onto proliferate (Pines and Hunter, 1990; Sherr, 1993). CD4 and CD8 cells were independently stimulated with *C. neoformans* and the expression of cyclin E and cyclin B1 were examined. Studies of cyclin expression have not previously been performed for isolated CD4 and CD8 cells. To determine the role of IL-2 in the CD8 cell response RT-PCR of IL-2 was performed, and by adding exogenous IL-2 to *C. neoformans*-stimulated CD8 cells. Finally, expression of CD25 and CD71 by CD4 and CD8 enriched

populations was assessed to determine whether these isolated populations could express T

cell growth factor receptors in response to *C. neoformans*.

3.2 RESULTS

3.2.1 T lymphocytes proliferate in response to *C. neoformans*

Before experiments analyzing individual T cell subsets were performed it was essential to determine whether T cells proliferated in response to *C. neoformans*, and also if other cells proliferated. To accomplish this the number of CD3 cells was determined at various times after stimulation with *C. neoformans*. PBMC were counted after various lengths of time in culture, and the percentage of cells that were CD3 positive was determined by flow cytometry. The number CD3 cells increased significantly over time in *C. neoformans* stimulated cultures from 154×10^3 to 233×10^3 , while the number of CD3 cells gradually decreased in unstimulated cultures from 154×10^3 to 81×10^3 (Table 2). After 9 days in culture the number of cells in stimulated cultures had increased significantly from the number of cells at day 0. Thus, CD3 positive cells proliferated in response to *C. neoformans*.

Having established that CD3 positive lymphocytes proliferated, experiments were performed to determine whether just T cells, but not other cells proliferated. For these experiments thymidine incorporation was determined in T cell enriched and T cell depleted populations. 2×10^5 *C. neoformans* were used for this and all subsequent stimulations.

This number of organisms gave good response with a minimal amount of variation (Table 3). PBMC were depleted of T cells by removing cells that bound to sheep red blood cells (rosette positive population), and retaining the rosette negative fraction. T cell enriched cells were obtained by isolating the rosette positive fraction. The T lymphocyte depleted

Table 2. The number of CD3 positive cells increases after stimulation with *C. neoformans*.

Number of CD3+ cells X 10 ³ ± sem*				
	Day 0	Day 5	Day 7	Day 9
Unstimulated	154.3±3.5	117.8±1.1	99.7±9.4	81.5±4.6
Stimulated	154.3±3.5	159.2±15.5	207.4±30.8§	233.4±29.5§†

*PBMC were stimulated with *C. neoformans* for 0, 5, 7, or 9 days. The number of cells was determined by counting the number of lymphocytes, and determining the percent CD3+ cells by flow cytometric analysis.

† p< 0.05 compared to day 0. §p<0.05 as compared to unstimulated cells. Mean of three experiments.

Table 3. Proliferative responses to varying amounts of *C. neoformans*.

Number of organisms	0 (n=12)	1X10 ⁵ (n=7)	2X10 ⁵ (n=12)	10X10 ⁵ (n=4)
cpm±sem x 10 ³ *	3.4±0.6	19.9±6.2	19.2±2.8	23.0±6.4

*PBMC were stimulated with acapsular *C. neoformans* for 7 days. Lymphocyte proliferation was determined by thymidine incorporation.

(rosette negative) population did not respond to *C. neoformans* even upon addition of irradiated T cells as a source of T cell help (Figure 1). Enriched T cells (rosette positive) did not respond to *C. neoformans* in the absence of irradiated cells indicating that they were depleted of accessory cells. The T cell enriched population (rosette positive); however, did respond to *C. neoformans* after addition of irradiated depleted cells. Depleted cells had <5% contaminating CD3 positive, and enriched cells were greater than 90% CD3 positive cells as assessed by staining and flow cytometry. These results suggest that T lymphocytes are necessary for the proliferative response to *C. neoformans* (Figure 1).

3.2.2 Activation of CD4 and CD8 cells in response to *C. neoformans*

Prior to proliferating T cells are activated. To determine which subset of T lymphocytes was activated in response to *C. neoformans* CD4 and CD8 cells were analyzed for expression of CD69, CD25 (IL-2R α) and CD71 (transferrin receptor). CD69 is an early inducible marker of T cell activation. It is expressed as early as one hour after stimulation and usually decreases by three days (Cebrian *et al.*, 1988). Initially, it was important to show that PBMC expressed CD69 in response to stimulation with *C. neoformans*. PBMC (2×10^5 /well) were stimulated with *C. neoformans* (2×10^5 /well). When PBMC were stimulated with *C. neoformans* CD69 expression was detectable at 4

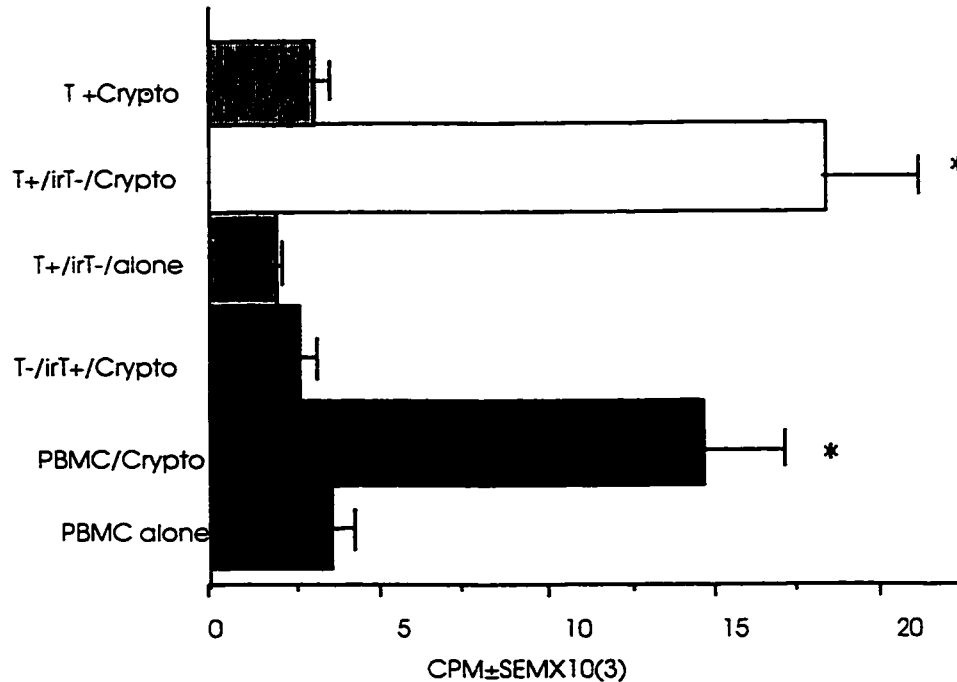


Figure 1: T lymphocytes proliferate in response to *C. neoformans*. PBMC were depleted of T lymphocytes (T⁻; 2X10⁵/well) or enriched for T lymphocytes (T⁺; 2X10⁵/well) by rosetting to SRBC and stimulated with *C. neoformans*. Irradiated (2500 rads), depleted T cells (irT⁻; 1X10⁵/well) or enriched T cells (irT⁺; 1X10⁵/well) were added back to the cultures. T depleted cells had <5% contaminating CD3⁺ cells. T enriched cells were >90% CD3⁺. *p < 0.05 compared to corresponding unstimulated cells. Lymphocyte proliferation was assessed 7 days later by thymidine incorporation. One of four representative experiments.

hours and continued to be expressed at low levels 48 hours later (Table 4). Due to low backgrounds and good responses a culture time of 4 hours was used for all subsequent experiments. To determine CD69 expression of T cell subsets PBMC were stimulated with *C. neoformans*. After four hours cells were harvested and examined for expression of CD69 on either CD4 or CD8 positive cells. When T cell subsets were examined for expression of CD69 it was found that the activation marker CD69 was expressed on both CD4 and CD8 cells after stimulation with *C. neoformans* (Table 5).

Expression of the T cell growth factor receptors CD25 and CD71 was examined. These growth factor receptors are required for cells to proliferate (Reed and Nowell, 1988). PBMC were stimulated with *C. neoformans*. At various times 4 wells were harvested and the CD4 and CD8 cells were analyzed for expression of the T cell growth factor receptors CD25 (IL-2R α) and CD71 (transferrin receptor). CD4 cells increased their expression of CD25 gradually from 8.8% on day 0 to 31.2% on day 9, and expression of CD71 from 1.7% on day 0 to 19.5% on day 9 (Table 6). CD8 cells did likewise increasing expression of CD25 from 2% on day 0 to 15.6% on day 9, and CD71 from 2.9% on day 0 to 16.2% on day 9 (Table 6). During stimulation with *C. neoformans* (day 0 to Day 9) the percentage of CD4 and CD8 cells expressing CD25 and CD71 increased between 3 and 10 fold. This indicated that both CD4 and CD8 cells were activated following stimulation with *C. neoformans* (Table 6).

Table 4: PBMC stimulated with *C. neoformans* express the T cell activation marker CD69.

		% PBMC that express CD69				
		<i>4 hours</i>	<i>8 hours</i>	<i>12 hours</i>	<i>24 hours</i>	<i>48 hours</i>
Unstimulated		6.3	10.3	20.1	7.1	8.1
Stimulated		14.2	16.0	26.5	9.8	9.2

One of three representative experiments.

Table 5: CD4 and CD8 cells stimulated with *C. neoformans* express CD69.

<i>Experiment</i>	<i>CD4/CD8</i>	<i>Unstimulated</i>	<i>Stimulated</i>
		<i>%CD69*</i>	<i>%CD69*</i>
1	CD4	4.4	13.3
	CD8	12.2	28.8
2	CD4	2.1	3.1
	CD8	10.1	10.3
3	CD4	7.2	15.0
	CD8	21.6	31.7
4	CD4	14.3	17.7
	CD8	14.4	15.2

* % of total CD4 or CD8 cells than express CD69

Table 6: CD4 and CD8 cells are activated in response to *C. neoformans*

		Percentage of CD4 or CD8 cells positive*			
		Day 0	Day 5	Day 7	Day 9
CD4	% CD25+	8.8±2.4	11.4±2.5	13.4±4.4	31.2±7.5†
CD4	% CD71+	1.7±0.4	6.1±1.6	9.8±2.1	19.5±2.5†
CD8	% CD25+	2.0±0.7	5.5±1.6	7.8±2.4	15.6±5.8†
CD8	% CD71+	2.9±1.4	7.6±1.1	4.8±2.2	16.2±5.8†

*PBMC were stimulated with *C. neoformans*, and at various times cells were harvested and analyzed for expression of CD25 or CD71 on CD4 or CD8 cells. Mean of four experiments.

† p<0.05 as compared to day 0

3.2.3 Phenotype of T lymphocytes responding to *C. neoformans*

Following stimulation, lymphocytes undergo a series of activation events and then enter the cell cycle. In some instances cells that are activated do not proliferate (Mazingue *et al.*, 1987; Paine *et al.*, 1991). This might explain a bias to expand one subset over another. It has also been demonstrated that lymphocyte activation and DNA synthesis can be followed by apoptosis rather than expansion (Iseki *et al.*, 1991; Lenardo, 1991; Groux *et al.*, 1992), and this can occur specifically in CD4 cells (Lopes *et al.*, 1995). If CD4 cells underwent cell death it may explain the previous observation of an increase in the proportion of cells expressing CD8 (Miller and Puck, 1984). This observation, however does not establish or exclude proliferation of either subset. To determine whether both CD4 and CD8 cells were capable of proliferating, PBMC were stimulated with *C. neoformans* and at various times the number of CD4 and CD8 cells was determined. The number of CD4 and CD8 cells increased indicating that both subsets proliferated in an unseparated population (Table 7). The absolute increase in the number of CD4 cells (8×10^4 cells/well) was greater than the increase in CD8 cells (3.6×10^4 cells/well). There was a two fold increase in the number of CD4 cells, and a 1.5 fold increase in CD8 cells over 9 days (Table 7) suggesting that CD4 cells have a greater contribution on a per cell basis than CD8 cells.

Table 7. The number of CD4 and CD8 cells increases after stimulation with *C. neoformans*.

		Number of cells/well \pm sem X 10 ³ *			
		Day 0	Day 5	Day 7	Day 9
CD4	Unstimulated	86.3 \pm 7.0	84.3 \pm 1.4	72.3 \pm 3.4	63.2 \pm 27.2
CD4	Stimulated	86.3 \pm 7.0	114.5 \pm 1.5†	139.7 \pm 9.4†	157.3 \pm 9.2†
CD8	Unstimulated	45.4 \pm 1.2	42.7 \pm 5.5	38.1 \pm 4.7	29.8 \pm 38.6
CD8	Stimulated	45.4 \pm 1.2	58.4 \pm 6.8†	77.2 \pm 7.8†	81.0 \pm 9.2†

*PBMC were cultured for 0, 5, 7 and 9 days with *C. neoformans*. The number of CD4 or CD8 cells was determined by counting the number of lymphocytes/well and determining the percent CD4 or CD8 positive by flow cytometry.

†p < 0.05 compared to Day 0. Mean of five experiments.

3.2.4 Contribution of CD4 and CD8 cells to the proliferative response.

Once it was established that CD4 and CD8 cells proliferated after stimulation with *C. neoformans* experiments were performed to determine the relative contribution of these subsets to thymidine incorporation. Treatment of PBMC with mAb to CD4 or CD8 block activation of each cell subset (Schmitt-Verhulst *et al.*, 1987; Swoboda *et al.*, 1987) without altering the number or ratio of cells in culture. Addition of anti-CD4 mAb to the culture abrogated proliferation to *C. neoformans*, while addition of anti-CD8 mAb caused a moderate reduction in proliferation (24% with 60 ng of anti-CD8, and 53% with 120 ng of anti-CD8) (Figure 2A). To confirm the specificity of anti-CD4 and anti-CD8 mAb tetanus toxoid (which would be expected to stimulate predominantly CD4 cells) and concanavalin A (Con A) (which would be expected to stimulate CD4 and CD8 cells) were used as controls (Carrera *et al.*, 1987; Schrezenmeier and Fleisher, 1988; Biselli *et al.*, 1992). Addition of 120 ng of anti-CD4 dropped proliferation in response to tetanus by 85% while 120 ng of anti-CD8 reduced proliferation by only 8%. Addition of both mAb decreased proliferation by 93% (Figure 2B). Addition of 120 ng anti-CD4 reduced proliferation in response to Con A by 39% while 120 ng of anti-CD8 reduced proliferation by 38% and addition of both mAb decreased proliferation by 97% (Figure 2C). These data demonstrates that both CD4 and CD8 cells contribute significantly to lymphocyte proliferation in response to *C. neoformans* as determined by thymidine incorporation.

A

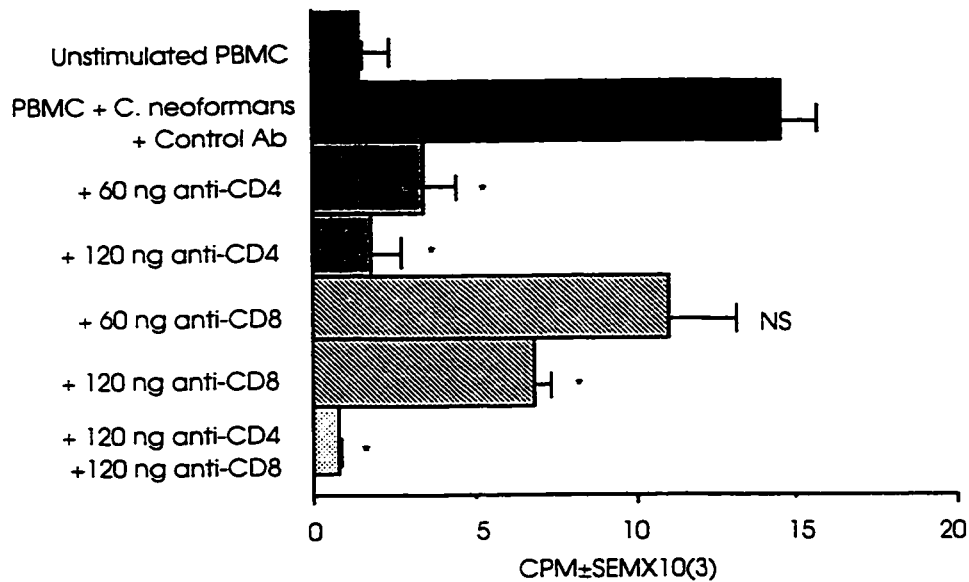


Figure 2A: Blocking CD4 and CD8 inhibits lymphocyte proliferation in response to *C. neoformans*. PBMC (2×10^5 /well) were unstimulated, stimulated with *C. neoformans* (2×10^5 /well) in the presence of 120 ng mouse IgG1 (control), or stimulated with *C. neoformans* in the presence of 60 or 120 ng of anti-CD4 or anti-CD8 mAb. Seven days later lymphocyte proliferation was assessed by thymidine incorporation

* $p < 0.05$ compared to stimulated PBMC in the presence of control antibody, NS - not significantly different than stimulated PBMC in the presence of control antibody. One of three representative experiments.

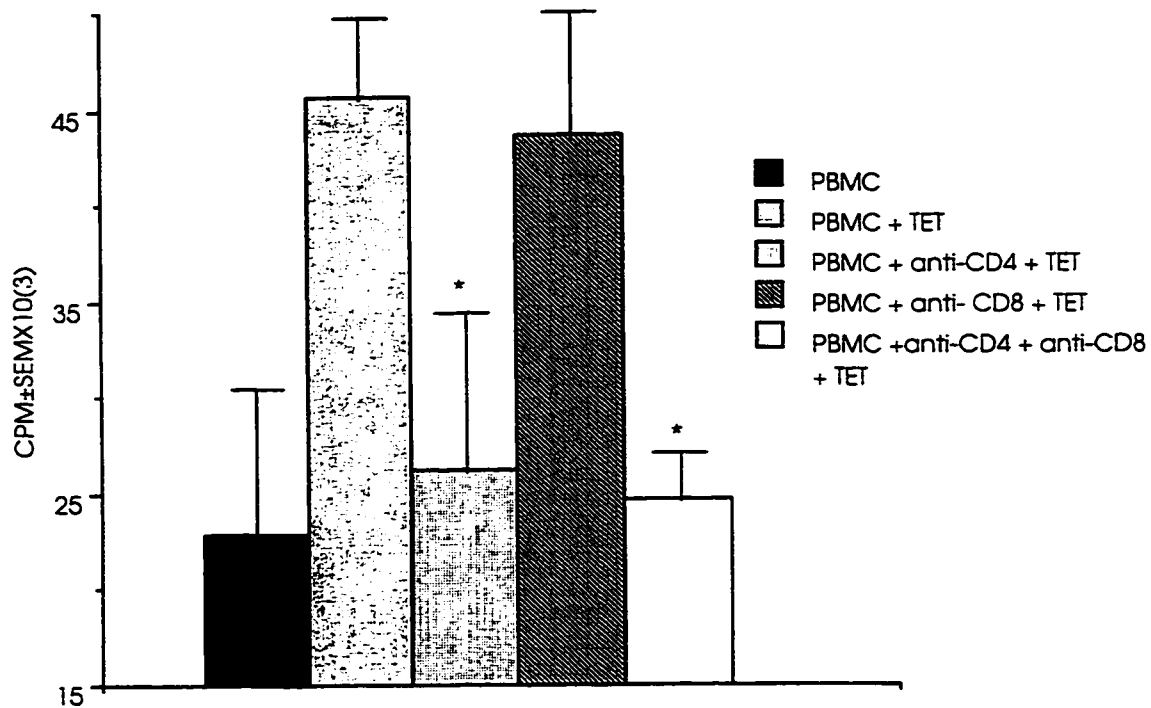
B

Figure 2B: Blocking CD4 inhibits lymphocyte proliferation in response to tetanus toxoid. PBMC (2×10^5 /well) were unstimulated, stimulated with tetanus (1:10 Lf U/ml) in the presence of 120 ng mouse IgG₁ (control), or stimulated in the presence of 120 ng of anti-CD4 or anti-CD8 mAb. Seven days later lymphocyte proliferation was assessed by thymidine incorporation. * $p < 0.05$ compared to stimulated PBMC in the presence of control antibody, NS - not significantly different than stimulated PBMC in the presence of control antibody. One of two experiments.

C

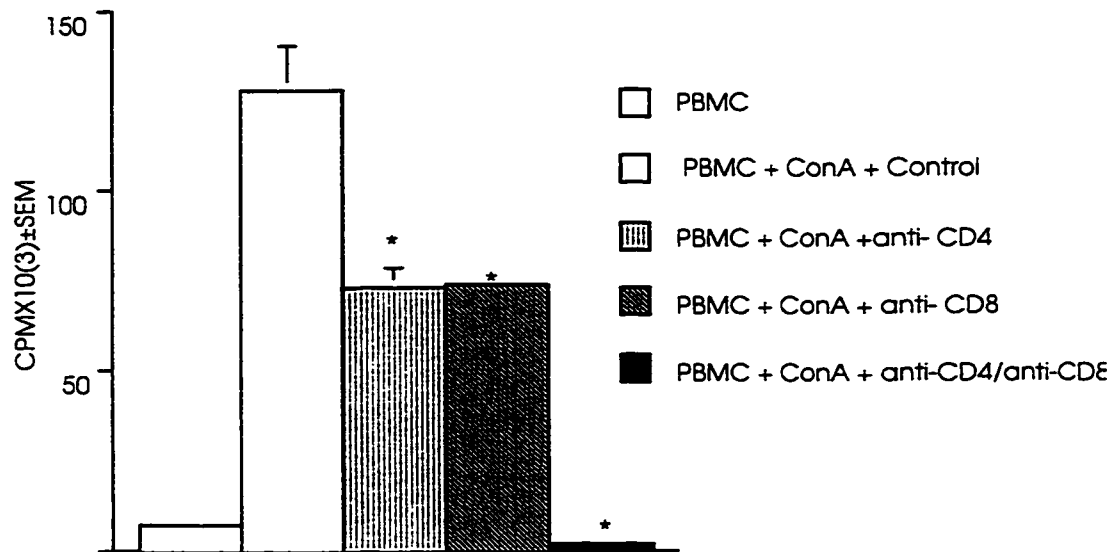


Figure 2C: Blocking CD4 and CD8 inhibits lymphocyte proliferation in response to Concanavalin A. PBMC (2×10^5 /well) were unstimulated, stimulated with Con A ($10 \mu\text{g/ml}$) in the presence of 120 ng mouse IgG1 (control), or stimulated in the presence of 120 ng of anti-CD4 or anti-CD8 mAb. Three days later lymphocyte proliferation was assessed by thymidine incorporation. * $p < 0.05$ compared to stimulated PBMC in the presence of control antibody. One of two experiments.

3.2.5 Presentation by MHC I and MHC II

Having determined that both CD4 and CD8 cells contribute to lymphocyte proliferation and proliferate in response to *C. neoformans* experiments were performed to determine if MHC II was used to present antigen to CD4 and CD8 cells, or whether both MHC I and MHC II molecules were used. Presentation of peptide antigens can occur to CD8 or CD4 cells by way of class I and class II MHC, respectively. Superantigens, however, are presented solely by MHC class II molecules to CD4 or CD8 cells (Fleisher *et al.*, 1988). It is possible that both exogenous and endogenous pathways that ultimately present antigen in conjunction with an MHC class II or class I molecules to CD4 and CD8, are utilized by *C. neoformans*, or the response may be like that to a superantigen. Experiments were performed to determine whether blocking class I or II MHC molecules abrogates cryptococcal induced lymphocyte proliferation. Antibodies against HLA-DR, DP and DQ caused a significant reduction in the response to *C. neoformans* (Figure 3A), as compared to responses in the presence of isotype matched control antibody. This suggests that the MHC II complex plays a critical role in the interaction required for T lymphocyte proliferation. Antibody against MHC Class I molecules also caused a significant reduction in lymphocyte proliferation (Figure 3A). This suggests that both types of MHC molecules are required for optimal presentation and ultimately T cell responses to the organism. Further, the percentage reduction caused by the anti-MHC antibodies was similar to the blocking caused when anti-CD4 and anti-CD8 antibodies

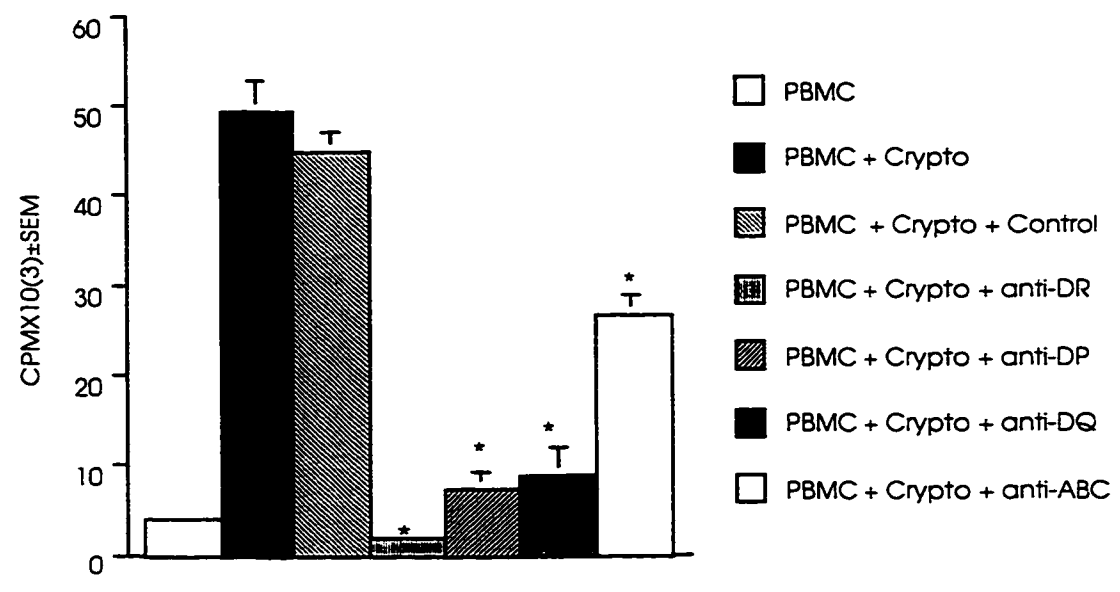
Figure 3A: Lymphocyte proliferation to *C. neoformans* is MHC class I and II dependent. PBMC (2×10^5 /well) were stimulate with *C. neoformans* (2×10^5 /well) in the presence of 120 ng of either anti-HLA-DR, anti-DP, anti-DQ, anti-ABC or isotype matched control antibody. Seven days later lymphocyte proliferation was assessed by thymidine incorporation.

* $p < 0.05$ as compared to isotype matched control. One of five representative experiments.

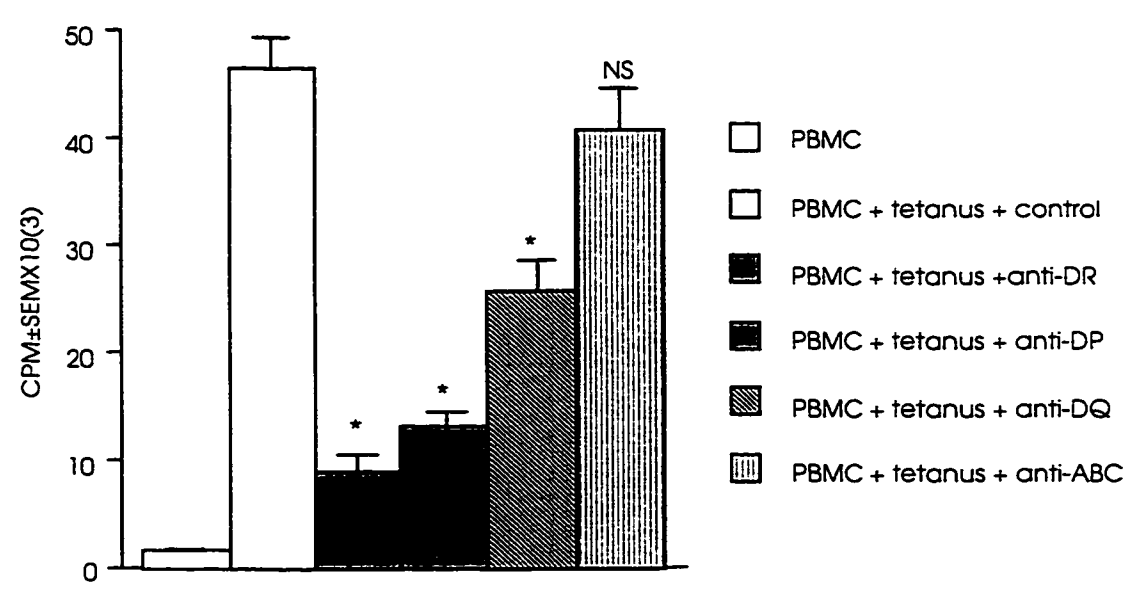
Figure 3B: Lymphocyte proliferation to tetanus toxoid is MHC class II dependent. PBMC (2×10^5 /well) were stimulated in the presence of 120 ng/ml of either anti-HLA-DR, anti-DP, anti-DQ, anti-ABC or isotype matched control antibody in the presence of tetanus toxoid 1:10 Lf. Seven days later lymphocyte proliferation was assessed by thymidine incorporation.

* $p < 0.05$ as compared to isotype matched control. NS- not significantly different as compared to control. One of three representative experiments.

3A



3B



were used. In control experiments, the proliferative response to tetanus toxoid, which activates CD4 cells, was blocked by class II MHC antibodies, but not by class I MHC antibody (Figure 3B).

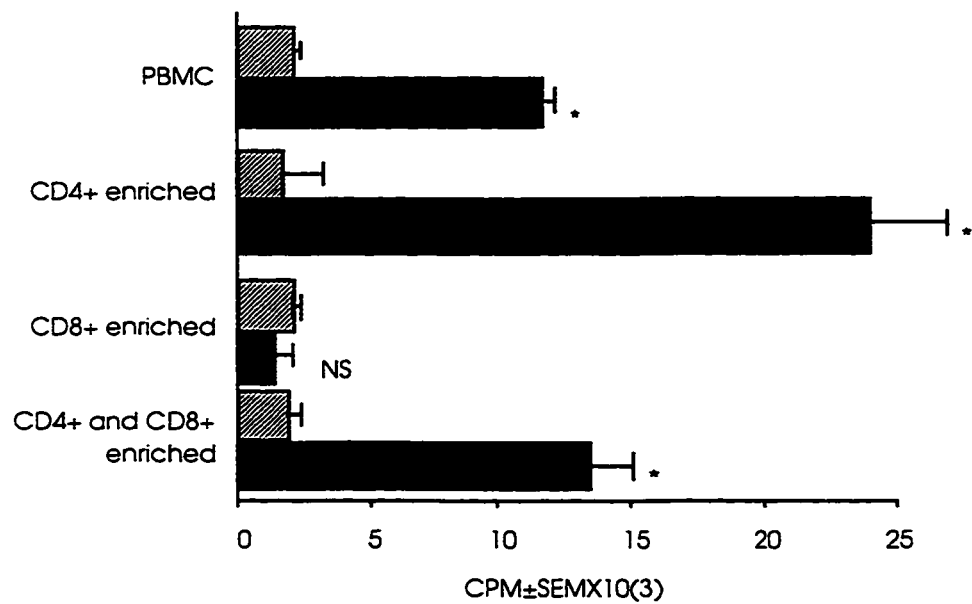
3.2.6 Requirement for CD4 or CD8 cells for the proliferative response

Since both CD4 and CD8 cells proliferate, and both produce cytokines that enhance lymphocyte proliferation (Seder *et al.*, 1988; Smith, 1988; Mosmann and Coffman, 1989), experiments were performed to determine whether either subset was necessary for the response to *C. neoformans*. PBMC were depleted of either CD4 or CD8 cells by immunomagnetic separation prior to stimulation with *C. neoformans*. CD8 depleted (CD4 enriched) cells exhibited an increase in proliferation compared with PBMC (Figure 4A) indicating that CD4 cells could proliferate in the absence of CD8 cells, and suggesting that CD8 cells contributed less to proliferation than CD4 cells. By contrast, CD4 depleted (CD8 enriched) cells did not proliferate, indicating that CD4 cells were necessary for proliferation of CD8 cells. Adding back CD4 enriched cells to CD8 enriched cells resulted in a response similar to that of unseparated cells. Thus, proliferation of CD8 cells was dependent on the presence of CD4 cells, and the contribution of CD4 cells to proliferation was greater than the contribution of CD8 cells. To ensure that both CD4 and CD8 cell populations were capable of proliferating, Con A served as a positive control. CD4 enriched cells were able to proliferate as well as unseparated PBMC to this T cell mitogen. CD8 enriched cells also proliferated, but at a reduced level (66% of the control) (Figure 4B).

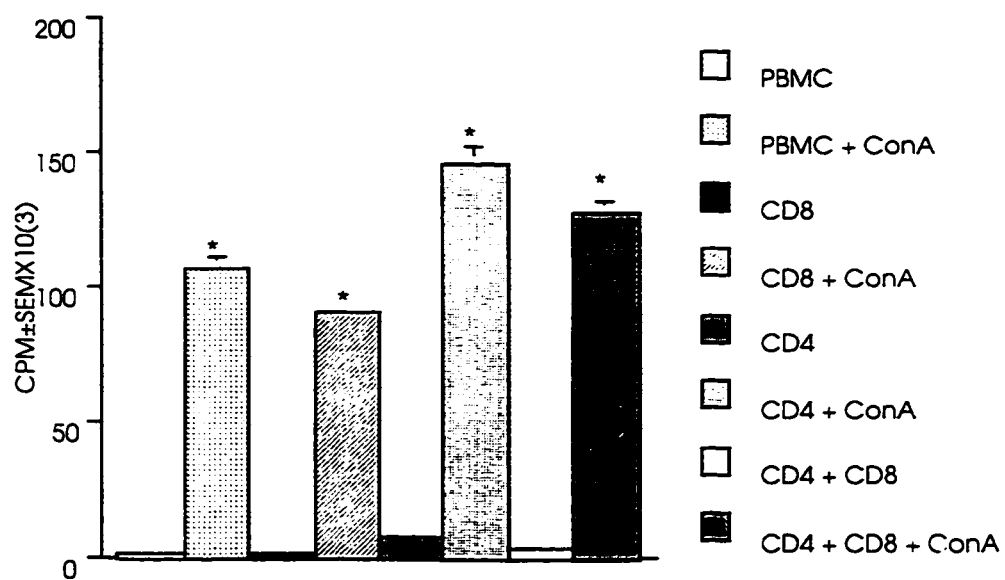
Figure 4A: CD8 cells require CD4 cells to proliferate in response to *C. neoformans*. PBMC, CD8 enriched, CD4 enriched or CD8 enriched added to CD4 enriched populations were stimulated with *C. neoformans*. (▨) Unstimulated (■) *C. neoformans*. All groups contained 2×10^5 cells/well. Seven days later lymphocyte proliferation was assessed by thymidine incorporation. Cells depleted of CD4 and CD8 had <2.5% of the contaminating population. * $p < 0.05$ compared to unstimulated cells. NS- not significantly different compared to unstimulated cells. One of five representative experiments.

Figure 4B: PBMC depleted of CD4 and CD8 cells proliferate in response to Con A. PBMC, CD4 depleted cells (CD8 enriched), or CD8 depleted cells (CD4 enriched) (2×10^5 cells/well) were stimulated with $10 \mu\text{g/ml}$ Con A. Three days later lymphocyte proliferation was assessed by thymidine incorporation. One of three representative experiments. * $p < 0.05$ as compared to stimulated unseparated PBMC
NS - not significantly different from unstimulated PBMC.

4A



4B



Having confirmed that both CD4 and CD8 cells contribute to lymphocyte proliferation, but CD8 cells are dependent upon CD4 cells for expansion, the response of CD8 cells to *C. neoformans* was examined. Previous studies were not clear as to whether CD4 and CD8 cells express activation markers at similar levels following various stimuli. In some instances, CD8 cells become activated and proceeded into cell cycle more rapidly than CD4 cells (Sprent and Schaefer, 1985), and in other circumstances, the products of CD4 cells are required for proliferation of CD8 cells (Selvan *et al.*, 1990). Since the pathways involved in presentation of cryptococcal antigen may be different than pathways used by Con A, viral antigens or alloantigens, the time course of activation in the subsets was determined by monitoring the expression of activation markers in response to *C. neoformans*.

3.2.7 Independent activation of CD8 cells

The ability of CD8 cells to be activated independently of CD4 cells was investigated by looking at CD69 expression on CD4 and CD8 cells. CD4 enriched or CD8 enriched cells were obtained by immunomagnetic depletion. Enriched populations were then stimulated with *C. neoformans*. As previous studies (Table 4) had established that optimal expression of CD69 occurred after 4 hours of stimulation with *C. neoformans* this time point was examined. In each population, T cells (CD3 positive) were analyzed for expression of CD69. In all experiments, both CD4 and CD8 depleted cells

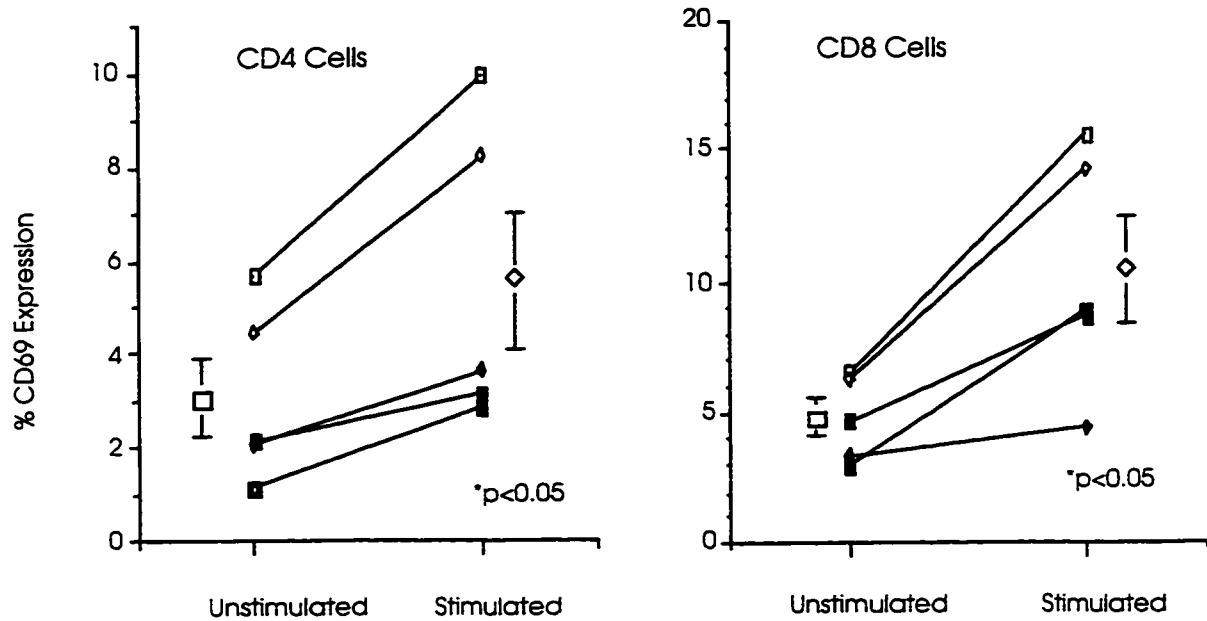


Figure 5: CD4 and CD8 cells are independently activated in response to *C. neoformans*. CD4 enriched or CD8 enriched cells (2×10^5 cells/well) were stimulated with *C. neoformans* (2×10^5 cells/well) for 4 hours. Cells were harvested from wells and then analyzed for expression of the early T cell activation marker CD69. The results from 5 separate experiments are shown. * $p < 0.05$ compared to unstimulated cells using a paired t test.

expressed increased levels of CD69 after stimulation with *C. neoformans* compared to unstimulated populations (Figure 5), indicating that *C. neoformans* independently activates both CD4 and CD8 cells. This suggests that CD4 cells do not contribute to the early activation of CD8 cells in response to *C. neoformans*, and that CD8 cells are able to respond to *C. neoformans* directly as they are able to express CD69 on their own.

3.2.8 CD8 cells independently enter cell cycle

Although CD69 expression indicates lymphocyte activation, it does not dictate that cells will enter cell cycle (Shenker and Datar, 1995). Further, in some instances cells that are activated do not go on to proliferate (Mazingue *et al.*, 1987; Paine *et al.*, 1989). To investigate whether independent CD8 cell activation was followed by cell cycle entry, the expression of genes that regulate the passage of cells through the cell cycle was studied. Cyclin E is expressed in G1 (Koff *et al.*, 1991) and indicates that cells have entered cell cycle. Cyclin B1 is expressed much later in the cell cycle, is required for the initiation of mitosis, (Jackman *et al.*, 1995), and suggests that cells are committed to the completion of S phase and mitosis. PBMC, CD4 enriched, and CD8 enriched cells were stimulated with *C. neoformans*. RNA expression of cyclins E and B1 was assessed by semi-quantitative RT-PCR (Wong *et al.*, 1994). Cells were harvested, RNA isolated and expression of these cyclins examined. PBMC upregulated expression of cyclin E and B1 in response to stimulation by *C. neoformans* (Figure 6). Upregulation of cyclin E was observed 72 hours after stimulation. Expression of B1 was observed after 48 hours in culture and was increased further after 72 hours. When CD4 and CD8 cell populations

6A

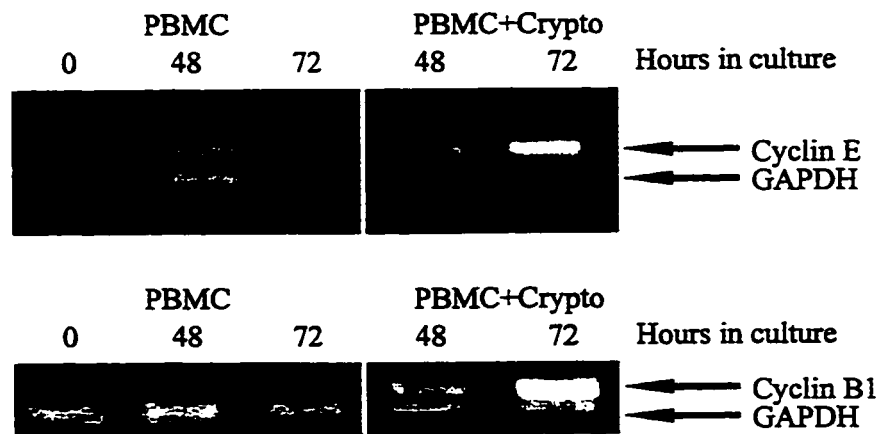
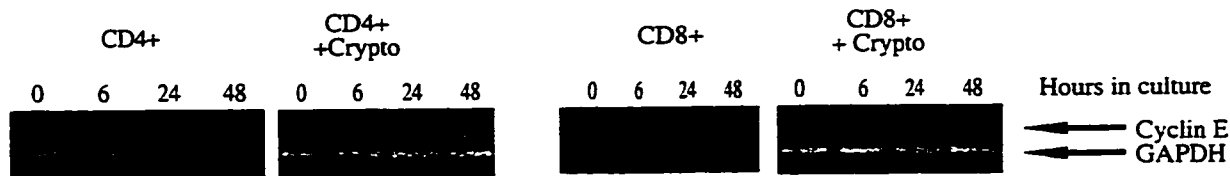


Figure 6: PBMC produce cyclin E, and cyclin B mRNA after stimulation with *C. neoformans*. PBMC (2×10^5 /well) were stimulated in the presence of *C. neoformans* (2×10^5 /well) for 0, 48 or 72 hours. At these times cells were harvested, RNA isolated and cyclin E (Panel A) or cyclin B1 (Panel B) were detected by RT-PCR. GAPDH was used as an internal control. The PCR product was visualized by ethidium bromide staining. The experiment was repeated with similar results.

7A



7B

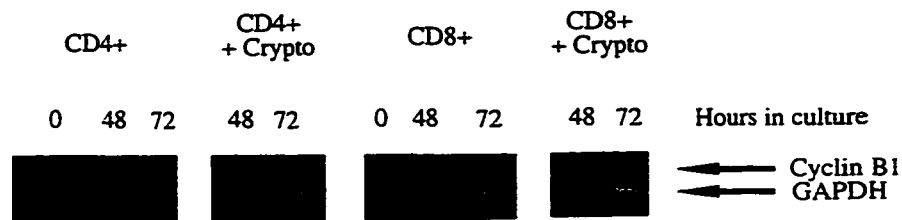


Figure 7: CD8 enriched cells express cyclin E mRNA, but fail to express cyclin B1 mRNA after stimulation with *C. neoformans*. CD8 or CD4 enriched cells (2×10^5 /well) were stimulated in the presence of *C. neoformans* (2×10^5 /well) for 0, 6, 24, 48, or 72 hours. At these times cells were harvested, RNA isolated and cyclin E or cyclin B1 detected by RT-PCR. GAPDH was used as an internal control. The PCR product was visualized by ethidium bromide staining. The experiment was repeated with similar results.

were stimulated with *C. neoformans* independently they both upregulated expression of cyclin E by 48 hours (Figure 7). When cyclin B1 expression was examined some notable differences occurred. CD4 cells expressed the cyclin B1 by 72 hours indicating that these cells progressed further through the cell cycle. By contrast, CD8 cells failed to express cyclin B1 after stimulation with *C. neoformans*, indicating that they were blocked in G1. Unstimulated cells failed to express cyclin B or upregulate cyclin E at any time point (Figure 6, 7). Thus, CD8 cells failed to progress through later stages of the cell cycle in the absence of CD4 cells

3.2.9 IL-2 mRNA production by isolated CD4 and CD8 cells

Failure of CD8 enriched cells to proliferate in response to *C. neoformans* may be due to deficient production of the T cell growth factor IL-2. To examine this possibility highly sensitive RT-PCR was used to detect expression of IL-2. CD4 and CD8 cells were examined for expression of IL-2 messenger RNA. PBMC, CD4 enriched, and CD8 enriched cells were stimulated with *C. neoformans* for 6, 24, or 48 hours. Cells were harvested and IL-2 mRNA expression was assessed by RT-PCR. As expected, PBMC expressed IL-2 mRNA by 24 hours. CD4 enriched cells also showed pronounced expression of IL-2 mRNA by 24 hours which was further upregulated by 48 hours. CD8 enriched cells, however failed to express IL-2 mRNA (Figure 8). Thus, CD8 cells failed to progress to later points in the cell cycle, and these CD8 enriched cells also failed to express IL-2 mRNA at all time points examined (Figure 8).

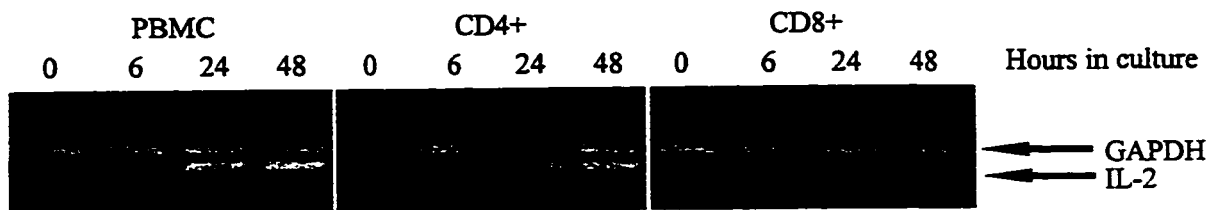


Figure 8: CD8 cells fail to produce IL-2 mRNA after stimulation with *C. neoformans*. CD4 enriched or CD8 enriched cells (2×10^5 /well) were stimulated with *C. neoformans* (2×10^5 /well) for 0, 6, 24 or 48 hours and then examined for expression of IL-2 mRNA by RT-PCR. GAPDH was used as an internal control. The PCR product was visualized by ethidium bromide staining. The experiment was repeated with similar results.

To determine whether it was a lack of IL-2 that was preventing CD8 cells from proliferating in response to *C. neoformans* CD8 enriched cells were put into culture with *C. neoformans* and various amounts of IL-2 (Gibco). The addition of this exogenous IL-2 did not allow CD8 cells to now go onto proliferate, even when high doses were used (Figure 9). PBMC proliferated in response to *C. neoformans* in the absence of exogenous IL-2. Proliferation increased when IL-2 was added at concentrations of 1 µg /ml, but also caused unstimulated cells to proliferate. CD8 enriched cells did not proliferate in response to *C. neoformans* and there was no increase in thymidine incorporation compared to unstimulated cells at any IL-2 concentration examined. This suggests that CD4 dependent signals in addition to IL-2 are required for CD8 cells to proliferate.

3.2.10 Expression of T cell growth factor receptors

T cell growth factors such as IL-2 can be supplied exogenously, but must bind to the appropriate receptor to activate CD8 cells. Therefore, expression of T cell growth factor receptors was assessed following stimulation with *C. neoformans*. Specifically, expression of the alpha chain of the IL-2 receptor (CD25), and the transferrin receptor (CD71) was examined. After 7 days of culture CD25 and CD71 were expressed by CD4 cells that were independently activated, but not by CD8 cells (Figure 10). This is in contrast to unseparated PBMC, where both CD4 and CD8 cells express CD25 and CD71 (Table 6). Only independently activated CD4 cells expressed CD25 and CD71. Thus, expression of the high affinity receptor for IL-2 and transferrin on CD8 cells required the presence of CD4 cells.

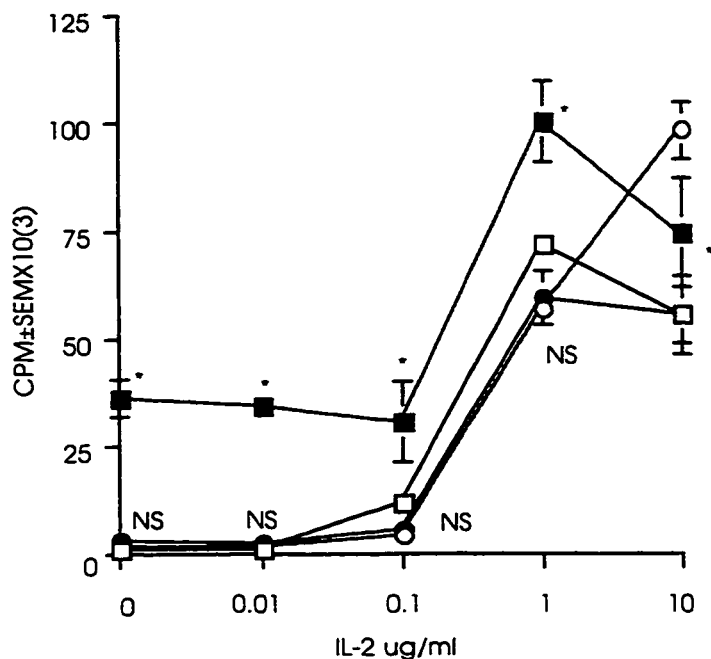


Figure 9: CD8 cells do not proliferate in response to *C. neoformans* even upon addition of IL-2. PBMC or CD8 enriched cells (2×10^5 /well) were put into culture in the presence or absence of *C. neoformans* (2×10^5 /well) with 0, 0.01, 0.1, 1 or 10 $\mu\text{g/ml}$ IL-2 for 7 days. Seven days later lymphocyte proliferation was assessed by thymidine incorporation. (□) PBMC, (■) PBMC + *C. neoformans*, (○) CD8 enriched cells, and (●) CD8 enriched cells + *C. neoformans*. * $p < 0.05$ as compared to corresponding control NS - not significant compared to corresponding control. The experiment was repeated with similar results.

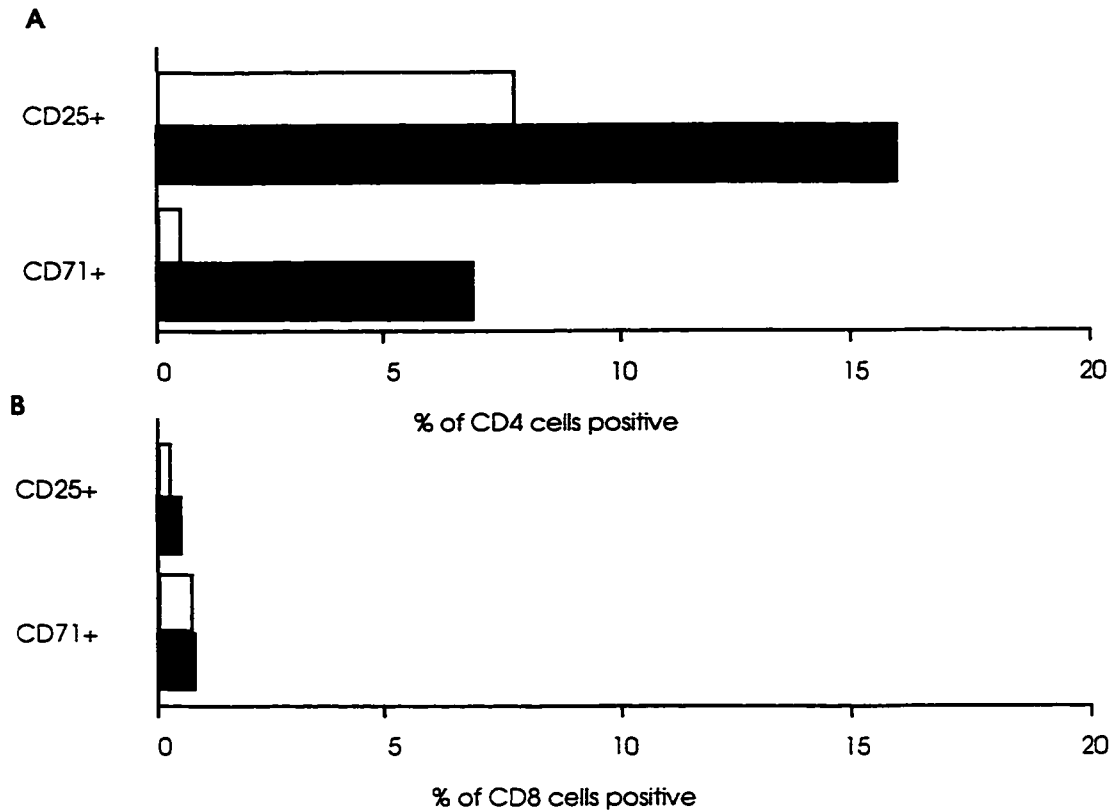


Figure 10: CD8 cells fail to express T cell growth factor receptors after stimulation with *C. neoformans*. PBMC were depleted of CD4 or CD8 cells. The depleted populations (2×10^5 /well) were stimulated with *C. neoformans* (2×10^5 /well). After 7 days in culture, expression of CD25 (IL-2R) and CD71 (transferrin R) were analyzed by flow cytometry in unstimulated (\square), or stimulated (\blacksquare) cells. One of three representative experiments.

3.3 DISCUSSION

Five observations have been made with regards to T cell subset responses to *C. neoformans*. 1) CD3 cells proliferate in response to *C. neoformans*. 2) Both CD4 and CD8 cells are activated and proliferate in response to *C. neoformans*. 3) CD4 cells are both necessary and sufficient for lymphocyte proliferation in response to *C. neoformans*. 4) CD8 cells are neither necessary nor sufficient, although they contribute significantly to the proliferative response. 5) CD8 cells were independently activated and entered the cell cycle, in response to *C. neoformans*, but required CD4 cells or their signals to express IL-2R α , progress through the cell cycle and proliferate.

To study the human immunologic response to *C. neoformans*, a model of *in vitro* lymphocyte proliferation has been developed (Miller and Puck, 1984; Mody and Syme, 1993). Lymphocytes from normal adults, who rarely become infected with *C. neoformans*, have a proliferative response to the organism, while HIV infected patients, that are at risk of cryptococcosis, have impaired proliferative responses (Hoy *et al.*, 1988). Since AIDS patients are predisposed to infections by *C. neoformans*, it suggests that the response of human lymphocytes may be important in defense of the organism, as it is in animal models (Mody *et al.*, 1990; Hill and Harmsen, 1991; Huffnagle *et al.*, 1991). These observations suggest that the *in vitro* proliferative response to *C. neoformans* correlates with protection in human disease. The current data demonstrates that CD4 cells are the most important subset for lymphocyte proliferation *in vitro* in response to *C. neoformans*. This is in keeping with the clinical association of *C. neoformans* infections

in AIDS patients with defective CD4 cell function, but in contrast to work by Miller and Puck (1984) who proposed that CD8 cells not CD4 cells proliferated.

Initially, T cell responses were examined to ensure that T cells expanded in response to *C. neoformans*, and further to ensure that they were the only responding population. Having established this, individual subsets were examined. T lymphocytes consist of two major subsets that have both been shown to enhance anticryptococcal host defense in animal models. Both CD4 and CD8 cells are necessary for optimal clearance of *C. neoformans* in mice (Mody *et al.*, 1990; Mody *et al.*, 1993). Both subsets participate in the murine inflammatory response (Hill, 1992; Huffnagle *et al.*, 1994) and in delayed type hypersensitivity (Fidel and Murphy, 1990; Mody *et al.*, 1994). The current experiments demonstrate that these murine models accurately reflect the human lymphocyte response to *C. neoformans*.

A number of possible explanations that could have accounted for previously described selective expansion of CD8 cells (Miller and Puck, 1984) were examined. Following activation, CD8 cells could contribute to proliferation and CD4 could become apoptotic (Schmitt-Verhulst *et al.*, 1987; Swoboda *et al.*, 1987; Lenardo *et al.*, 1991; Groux *et al.*, 1992; Lopes *et al.*, 1995). CD4 cells could also become activated, not proliferate, but release factors that allow CD8 cells to proliferate. Thus, the number of CD4 and CD8 cells after activation with *C. neoformans* was studied to determine whether they increased, indicating proliferation, or decreased suggesting apoptosis. The previous observation that CD8 cells proliferated was confirmed, but for the first time it was established that CD4 cells also proliferate in response to *C. neoformans*. Indeed, the

increase in the number of CD4 cells was greater than the increase in the number of CD8 cells. CD4 enriched cells proliferated more vigorously than the same number of PBMC, suggesting that each CD4 cell contributed more to proliferation than each CD8 cell.

AIDS patients have defective CD4 cell responses and are predisposed to cryptococcal infections. This suggests that CD4 cells play an essential role *in vivo*. Experiments were undertaken to determine whether CD8 cells could proliferate independently, or whether they required the contribution of CD4 cells. By depleting PBMC of either the CD4 or CD8 subset and comparing the proliferative response, it was demonstrated that CD4 cells were required for proliferation to *C. neoformans*. CD8 cells were unable to proliferate on their own. Experiments using mAb to remove the contribution of the selected cells while preserving the number and ratio of CD4 and CD8 cells, which may be critically important in lymphocyte proliferation were performed. Use of mAb to CD4 or CD8, which block proliferation in response to phorbol esters and calcium ionophore, indicated that they block proliferation independent of the mechanism of activation (Schmitt-Verhulst *et al.*, 1987; Swoboda *et al.*, 1987). These experiments confirmed that CD4 cells are required and are the major contributor to lymphocyte proliferation, since proliferation was abrogated by blocking CD4 cells.

Proliferation of CD4 cells could have two possible mechanisms by which it could enhance host defense to *C. neoformans*. First, CD4 proliferation would expand the population of cytokine-producing T lymphocytes that stimulate anticryptococcal effector cells including NK cells (Murphy and McDaniel, 1982), CD8 cells (Levitz, 1991b) and macrophages (Mody *et al.*, 1991; Chen *et al.*, 1994). Second, proliferation would enhance

host defense by expanding CD4 cells, which have direct anticryptococcal growth inhibitory activity (Levitz *et al.*, 1994b). These studies also confirmed the previous observation that CD8 cells contribute to the response as blocking them caused a reduction in lymphocyte proliferation.

These studies also suggest that *C. neoformans* stimulates lymphocyte proliferation by more than one mechanism. Following stimulation with *C. neoformans*, CD8 cells expressed IL-2R α , transferrin receptor and proliferated. This raises the possibility that *C. neoformans* is presented by both class I MHC to CD8 cells and class II MHC to CD4 cells. Since antigen presentation to CD8 cells is via class I MHC and the endogenous pathway, and presentation to CD4 cells is usually via class II MHC and the exogenous pathway (Germain 1986; Morrison *et al.*, 1986), it suggests that *C. neoformans* antigens gain access to both antigen processing pathways. Additionally, protein released during extracellular killing may gain access to exogenous pathways to activate CD4 cells (Allen *et al.*, 1987). Organisms that gain access to the intracellular space can be processed by endogenous antigen processing pathways (Turner and Dockrell, 1996). Since *C. neoformans* can gain access to the intracellular space, has been reported to survive intracellularly and become extra-phagosomal (Herrmann *et al.*, 1994; Lee *et al.*, 1995), it is possible that cryptococcal antigens may gain access to the intracellular compartment and be processed by the endogenous pathway, displayed on MHC class I, and presented to CD8 cells (Townsend and Bodmer, 1989). Alternately, the possibility of a mitogenic effect as proposed by Graybill (Graybill and Alford, 1974), which could activate both CD4 and CD8 cells has not been excluded. MHC blocking experiments provide some

evidence that the proliferative response is antigenic rather than mitogenic. As well, blocking experiments demonstrated that MHC I and II molecules were used for this activation of CD4 and CD8 cells, suggesting that the organism somehow gained access to the endogenous pathway of presentation, rather than used MHC II molecules to present to CD8 cells.

Since CD8 cells did not proliferate independently, it was important to determine whether CD8 cells were independently activated in response to *C. neoformans*, or whether they were non-specifically activated by responding CD4 cells. Initially, it was established that unseparated CD4 and CD8 T cell subsets both expressed the activation markers CD69 (an early T cell activation marker), CD25 (interleukin-2 receptor α) and CD71 (transferrin receptor). These markers indicate cellular activation (Reed *et al.*, 1986; Waldman, 1991) and are necessary for lymphocytes to proceed through the cell cycle (Reed and Nowell, 1988).

Having established that CD4 and CD8 cells expressed detectable levels of activation markers the independent activation of these subsets was examined. Isolated CD8 cells expressed CD69 indicating these cells can be “triggered” by *C. neoformans*. These are the first experiments to demonstrate that CD8 cells can be activated independently of CD4 cells in response to *C. neoformans*.

Expression of CD69, however, does not necessarily mean that cells will enter the cell cycle (Shenker and Datar, 1995). Therefore, the expression of cyclins, which control the kinases that drive the cell cycle in response to growth factor-mediated signals was examined. Eukaryotic cells synthesize multiple cyclins that appear at different times

during the cell cycle. Cyclin E complexes with Cdk2, forming an active kinase in mid G1 with maximal activity in late G1 (Dulic *et al.*, 1992; Koff *et al.*, 1992). Cyclin E expression is required for progression through G1 into S phase (Koff *et al.*, 1993; Ohtsubo and Roberts, 1993). Its expression is prior to the restriction point of the cell cycle (Ohtsubo and Roberts, 1993). Cyclin B1 is expressed later than cyclin E. Cyclin B1 also complexes to Cdk2 and requires active cyclin E/Cdk2 for expression (Dulic *et al.*, 1993). Complexes of Cdk2 with cyclin E can activate the cyclin B1 promoter (Katula *et al.*, 1997). Cyclin B1 appears during S phase (Pines and Hunter 1992), and is involved in the initiation of mitosis (Jackman *et al.*, 1995). CD4 cells and CD8 cells expressed cyclin E following stimulation with *C. neoformans*. However, CD4 cells, but not CD8 cells, expressed cyclin B1. This is the first demonstration of cyclin gene expression in purified CD4 and CD8 cells, and suggests that both T cell subsets independently enter the cell cycle, but that only CD4 cells express the cyclins necessary to proceed through G1 and S phases, and subsequently through mitosis.

Growth factors are critical for cyclin expression in many cell systems (Matsushime *et al.*, 1991; Tsai *et al.*, 1993). Interleukin 2 is an important growth factor for progression of T lymphocytes through cell cycle. Addition of IL-2 has been reported to stimulate Cdk2 expression (Modiano *et al.*, 1994), and to inactivate an inhibitor of Cdk2 in T cells (Firpo *et al.*, 1994), which allows kinase activity. This is associated with increased levels of cyclin B and subsequent progression to M phase following stimulation with phytohemagglutinin (PHA) (Firpo *et al.*, 1994). Experiments were performed to determine whether *C. neoformans* stimulated CD8 cells expressed IL-2, since inadequate

levels of IL-2 could produce this effect. Isolated CD4 cells were found to express IL-2 mRNA, while CD8 cells did not.

In order for IL-2 to be effective, it must bind to the IL-2 receptor (Cantrell and Smith, 1984; Stern and Smith, 1986). CD8 cells respond and proliferate in response to antigens, but have a limited capacity to produce IL-2, which provides an essential T cell growth factor for proliferation, and often require the participation of CD4 cells. This study suggests that CD4-dependent IL-2 production is likely to be one mechanism that CD4 cells are required for CD8 cell proliferation. Another CD4 dependent signal must also be required as restoration of proliferation was not accomplished by the addition of exogenous IL-2. IL-2R α can be expressed independently of IL-2 (Kumagai *et al.*, 1987; Crabtree, 1989). This has been demonstrated after cells were stimulated with TNF- α (Lee *et al.*, 1987), which is produced by *C. neoformans*-stimulated PBMC (Levitz *et al.*, 1994a). Therefore, the possibility that *C. neoformans* might stimulate expression of IL-2R α was considered. If IL-2R α were expressed, then addition of exogenous IL-2 might restore the response. Expression of the transferrin receptor (CD71) was also examined, since binding of transferrin to its receptor is required for T cells to undergo G1-S phase transition (Neckers and Cossman, 1983). CD4 cells were found to express IL-2R α , while CD8 cells did not. The same pattern was observed for the transferrin receptor. Expression of CD71 on CD8 cells was not expected, as IL-2 is involved in the expression of CD71 (Neckers and Cossman, 1983; Reed and Nowell, 1986). These observations are consistent with previous studies in which unseparated, PHA-stimulated PBMC that were IL-2R α positive expressed cyclin A while IL-2R α negative cells did not (Firpo *et al.*,

1994). Cyclin A is expressed after cyclin E and before B1 (Sherr, 1993). Since isolated CD8 cells failed to express T cell growth factor receptors (IL-2R α and transferrin receptor), it is unlikely that replacement of IL-2 alone would restore CD8 cell proliferation. The CD4 dependent signal required is likely to be proximal to IL-2 signaling. These CD4 dependent signals must be provided if CD8 cells are to participate in the human lymphocyte response to *C. neoformans*.

In summary, a crucial role for CD4 cells in the lymphocyte response to *C. neoformans in vitro* has been demonstrated. This is the first experimental evidence to suggest that human CD4 cells participate in the afferent immunologic response to *C. neoformans*. Further, CD8 cells have been shown to make a significant contribution to the T lymphocyte proliferation in response to *C. neoformans* and can be activated and enter the cell cycle independent of CD4 cells, but fail to progress and express M phase cyclins, which are necessary for DNA synthesis. This suggests that initial antigen presentation to CD8 cells is intact and that subsequent CD4 dependent signals are required to complete the CD8 cell response. Accordingly, if the appropriate CD4-derived signals could be identified, perhaps CD8 cells could become fully activated, which would be an important addition to host defense to *C. neoformans*. These CD8 cells could function as cytotoxic cells for *C. neoformans* (Levitz and Dupont, 1993), or release cytokines such as γ -IFN (Salgame *et al.*, 1991), which would activate cells for anti-cryptococcal activity (Mody *et al.*, 1991; Salkowski and Balish, 1991; Murphy, 1993). Thus, these CD4-derived signals may be a valuable therapeutic adjunct in AIDS patients infected with *C. neoformans*.

CHAPTER 4

THE ANTIGEN PRESENTING CELL

4.1 BACKGROUND

Information regarding the antigen presenting cell requirements of *C. neoformans* is very limited. Rather than investigating the cell type most studies have inferred that the most important APC for *C. neoformans* is the highly phagocytic macrophage (Collins and Bancroft, 1991; Vecchiarelli *et al.*, 1994a, 1994b). This seems likely based on the characteristics of the organism. First, the organism is relatively large when compared to pathogenic bacteria, or viruses that are engulfed by phagocytes. Prokaryotes are usually 1 μm in diameter, while *C. neoformans* ranges from 4-12 μm in diameter with a polysaccharide capsule that can be as large as 30 μm (Perfect, 1989; Levitz, 1991a). The size of an organism can limit both the uptake mechanisms available, as well as the cells that can accommodate them (Unanue, 1984; Brown, 1995; Lanzavecchia, 1996). Secondly, *C. neoformans* has a sturdy cell wall. The cell wall of *C. neoformans* consists of a matrix of carbohydrates such as glucan, N-acetylglucosamine and mannose in the form of mannoproteins (Reiss *et al.*, 1986; Vartivarian *et al.*, 1989). Complex carbohydrates, like those in the cell wall of *C. neoformans*, have been speculated to interfere with antigen processing (Gonzalez-Fernandez *et al.*, 1997). It is for these

reasons that it has been speculated that such a large microbe would demand a robust highly phagocytic APC.

The nature of the antigen presenting cell is integral in determining the outcome of the immune response. For example, antigen presentation by a B cell or a macrophage can in part determine whether a Th1 or a Th2 response is generated (Paul and Seder, 1994). APC type can also effect the immunodominant peptides generated, and thus influence the responding T cell population (Gapin *et al.*, 1998). Such differences are felt to be based upon the unique features of various APC populations such as receptor expression, processing machinery available, and costimulatory and accessory molecule expression. Different APC may express different receptors for antigen uptake or use different uptake pathways, which will bias them toward or against certain antigens. For instance, dendritic cells can internalize mannosylated antigens by mannose and lectin like receptors, and small particulates via macropinocytosis (Sallusto *et al.*, 1995). Antigen specific B cells use surface-expressed antigen specific immunoglobulin (Bonnerot *et al.*, 1995), while highly phagocytic monocytes and macrophages can utilize FcR, CR and receptors for a variety of polysaccharides (Unanue and Allen, 1987; Stahl, 1992). The uptake mechanism determines the intracellular compartment to which antigen is delivered guiding the antigen to early endosomes or MHC. The receptors involved can then directly effect the type of T cell epitopes generated by altering susceptibility to processing (Jacquier-Sarlin *et al.*, 1995). Different APC have also been demonstrated to have different abilities to process antigen, possibly due to differences in protease content (Vidard *et al.*, 1992; Schneider and Sercarz, 1998). Compartments where peptides are

processed and loaded onto MHC molecules may also vary, as demonstrated by the different processing pathways involved in presentation of distinct HEL epitopes (Zhong *et al.*, 1997). Taken together these differences lead to different epitopes being generated and presented to T cells (Vidard *et al.*, 1992; Sallusto *et al.*, 1995, Zhong *et al.*, 1997; Gapin *et al.*, 1998), which would determine the responding T cell population. Expression of costimulatory and accessory molecules also varies between APC, and could also influence the outcome of a T cell response (Gajewski *et al.*, 1991; Thompson, 1995).

The following studies were undertaken to examine the antigen presenting cell requirement of *C. neoformans*. To date no studies have systematically examined the cell population involved in the generation of T cell responses to *C. neoformans*. Initial experiments investigated whether an antigen presenting cell was required for initiation of a response. Subsequently, a systematic approach to identifying the cell that was employed. Functional properties as well as phenotypic differences in APC populations were used as a method of isolating individual cell populations. The cell was then further characterized by examining its morphology, phenotype and interactions with *C. neoformans*. Although blood/tissue derived antigen presenting cells are far more likely to be important in the generation of cell mediated immunity, the first contact of *C. neoformans* with phagocytic cells is in the alveolar space. Hence, cells obtained by bronchoalveolar lavage also were assessed.

Once the antigen presenting cell was identified the mechanism of attachment was investigated between this large microbe and its antigen presenting cell. Dendritic cells can bind antigen via FcR (Sallusto and Lanzavecchia, 1994), hydrophobic interactions

(macropinocytosis), and mannose receptors (Sallusto *et al.*, 1995). Macropinocytosis usually results in vesicles of only 1-3 μm in size (Lanzavecchia, 1996); therefore, it seemed unlikely this method of uptake could accommodate *C. neoformans*.

Consequently a receptor mediated event was pursued. Endocytosis of mannosylated ligands is well documented and can be mediated through the mannose receptor (Engering *et al.*, 1997; Tan *et al.*, 1997). It seems plausible then that dendritic cells could take up the pathogen by the mannose receptor. Since the cell wall of *C. neoformans* consists of carbohydrates such as glucan, N-acetylglucosamine and mannose (Reiss *et al.*, 1986) studies were performed to determine whether this newly described pathway could be involved in the interaction between dendritic cells and *C. neoformans*.

It has previously been shown that Langerhans cells use the mannose receptor to recognize mannans present on bacterial and yeast zymosan (Reis e Sousa *et al.*, 1993). Further, ingestion of acapsular *C. neoformans* has been shown to occur via mannose and β -glucan receptors of macrophages (Cross and Bancroft, 1995). Although there are a number of reports on antigen uptake via the mannose receptor (Engering *et al.*, 1997; Tan *et al.*, 1997; Cross and Bancroft, 1995), only one report has connected this mechanism of uptake with presentation to T cells during an immune response (Prigozy *et al.*, 1997). In this instance mycobacterium lipoarboromannan was presented to T cells in context of CD1. There is no information on whether microorganisms taken up by this novel pathway can ultimately be presented to T cells, or whether use of this receptor results in MHC I and MHC II presentation.

To determine the APC responsible for presentation of *C. neoformans* a series of purifications were performed. Previous information on the adherent characteristics of APC was used to separate cells based on adherence to plastic. To determine whether cells with cytolytic granules were required, treatment with leucine methyl ester was used to deplete this population. Surface phenotype was examined by sequential immunomagnetic separation. The cell was then characterized by its morphology, surface phenotype, and function. Finally, the mechanism of uptake of *C. neoformans* that was required for antigen presentation was examined by determining the contribution of the mannose receptor to antigen processing.

4.2 RESULTS

4.2.1 Antigen presenting cell requirement

Recall antigens require an APC to stimulate T lymphocytes to proliferate. Recall antigens must be taken up by the antigen presenting cell, processed and placed in the antigen binding site of the MHC molecule for presentation to the appropriate T cell.

To confirm that the T cell response to *C. neoformans* requires an antigen presenting cell, purified T cells were stimulated with *C. neoformans* in the presence or absence of antigen presenting cells. T cells were greater than 98% CD3 positive as determined by flow cytometry. They were unable to respond to a T cell mitogen, Con A, in the absence of an antigen presenting cell (control group for Figure 12B). Initially, unseparated irradiated PBMC were used as an APC. Cells were irradiated so that they were unable to proliferate, but still fully capable of processing and presentation (Germain, 1981). Irradiated APC, without T cells, put into culture with *C. neoformans* never elicited a proliferative response. T cells were unable to respond to *C. neoformans* in the absence of APC, but were capable of significant lymphocyte proliferation upon the addition of irradiated APC (Figure 11). Thus, T cells require an APC for presentation of *C. neoformans*.

4.2.2 The APC for *C. neoformans* is loosely adherent

Having determined that T cells respond to *C. neoformans* and require an APC experiments were performed to determine the cell type of the APC. In order to assess the best APC source for lymphocyte proliferation in response to *C. neoformans* a

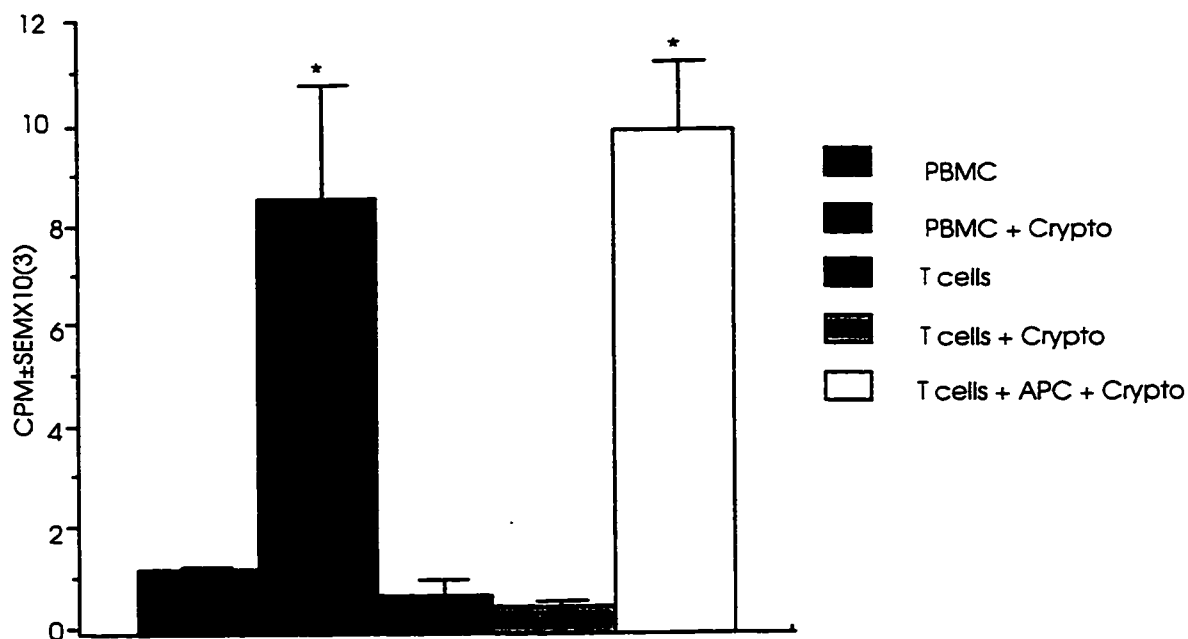


Figure 11: An antigen presenting cell is required for T-lymphocyte proliferation to *C. neoformans*.

Purified T cells (2×10^5 /well) were stimulated by *C. neoformans* (2×10^5 /well) in the presence of absence of irradiated PBMC (APC; 1×10^5 /well) as a source of APC. Seven days later lymphocyte proliferation was assessed by thymidine incorporation. One of four representative experiments.

* $p < 0.05$ as compared to unstimulated cells.

series of purifications were undertaken. Initially, cell populations were investigated based on their adherence properties. This technique has previously been used to separate subsets of APC populations (Steinman and Cohn, 1974). Steinman and Cohn had determined that monocytes were firmly adherent, while dendritic cells were loosely adherent. This technique enriched for two important antigen presenting cell populations, with minimal cellular manipulation and no addition of exogenous agents. Experiments were performed to assess the APC function of cells that were firmly adherent to plastic petri dishes. These firmly adherent cells were found to be a poor source of APC (Figure 12A) as compared to unseparated irradiated PBMC. These cells, however, were capable of acting as APC for T cell responses to a T cell mitogen, concanavalin A (Figure 12B) and were much more potent than irradiated PBMC, suggesting that a monocyte enriched population was more effective for this response. Upon phenotypic examination, this adherent population of cells was found to be enriched for CD14⁺ and CD56⁺ cells and depleted of CD3 and CD19 positive cells (Table 8), as has previously been described (Steinman and Cohn, 1974). Thus, these data suggested that monocytes were a poor source of APC for T cell responses to *C. neoformans*. This was surprising as most studies have assumed that *Cryptococcus* would be processed and presented by a highly phagocytic monocyte/macrophage (Collins and Bancroft, 1991; Vecchiarelli *et al.*, 1994a, 1994b).

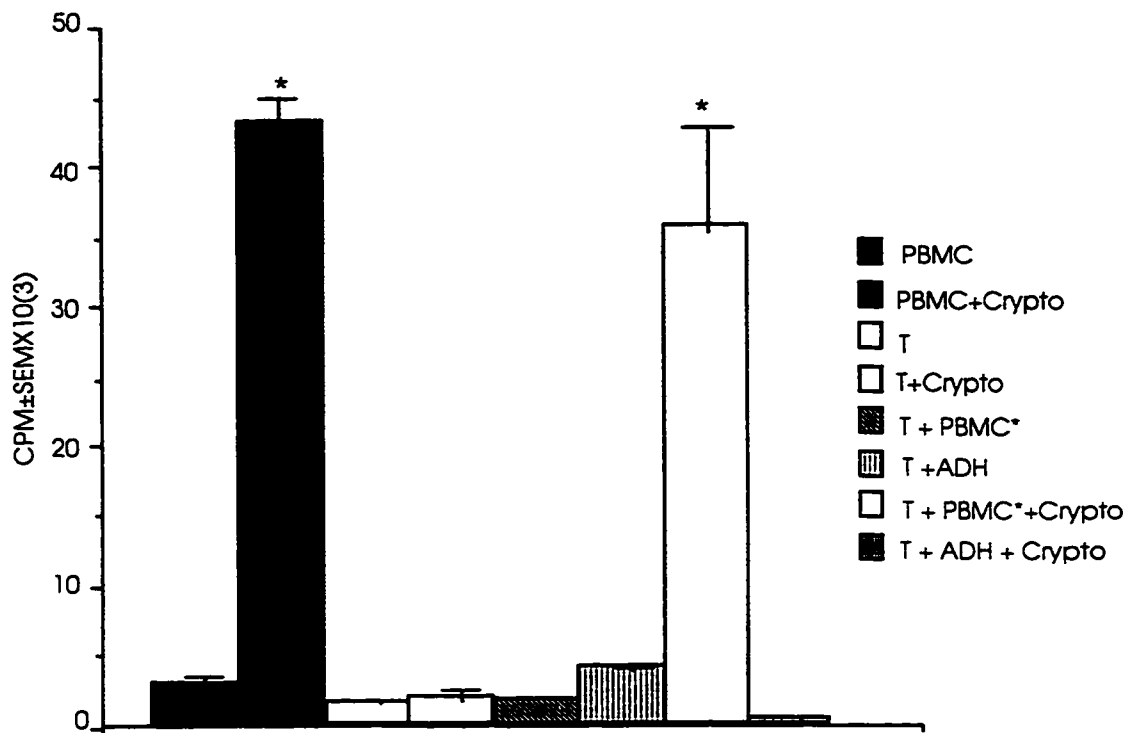
Loosely adherent cells have been described as cells that are adherent to plastic after a two hour incubation and nonadherent after a further overnight incubation. Further, they are described as being enriched for dendritic cells, a potent APC population (Van

Figure 12A: Firmly adherent cells are a poor source of antigen presenting cells for *C. neoformans*. T cells (2×10^5 /well) were put into culture with either irradiated PBMC (PBMC*; 1×10^5 /well), or irradiated firmly adherent cells (ADH; 1×10^5 /well) as a source of APC, in the presence or absence of *C. neoformans* (2×10^5 /well). Seven days later lymphocyte proliferation was assessed by thymidine incorporation.. * $p < 0.05$ as compared to corresponding unstimulated control. One of four representative experiments

Figure 12B: Firmly adherent cells can act as antigen presenting cells for a T cell mitogen, Con A. T cells (2×10^5 /well) were put into culture with either irradiated PBMC (PBMC*; 1×10^5 /well) or irradiated firmly adherent cells (ADH; 1×10^5 /well) as a source of APC, in the presence or absence of Con A ($2.5 \mu\text{g/ml}$). Three days later lymphocyte proliferation was assessed by thymidine incorporation. * $p < 0.05$ as compared to unstimulated T cells + corresponding APC.

One of four representative experiments.

12A



12B

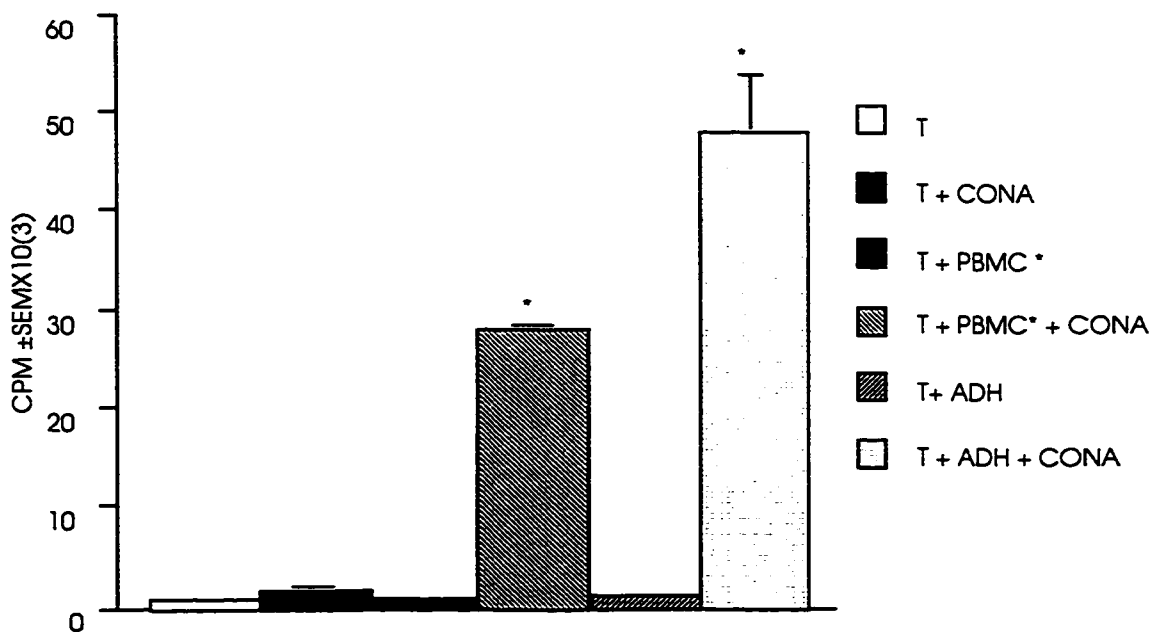


Table 8: Phenotype of cells isolated on the basis of adherence to plastic

<i>Cell marker</i>	% cells expressing surface marker ±sem		
	<i>Unseparated</i>	<i>Firmly Adherent*</i>	<i>Loosely Adherent*</i>
	<i>PBMC*</i>		
CD14	9.7±1.8	45.6±4.9	15.9±2.9
CD19	10.8±1.3	5.7±2.1	14.9±5.2
CD3	70.2±3.1	39.3±5.6	18.1±3.3
CD56	11.4±1.3	17.1±0.6	11.4±3.4
HLA-DR	26.0±2.6	32.5±18.4	60.0±6.0

*Mean of 5 experiments.

Voorhis *et al.*, 1982). Loosely adherent cells were found to be a potent source of APC for lymphocyte proliferation to *C. neoformans* (Figure 13). They were much better than irradiated PBMC at inducing T cell proliferation in response to *C. neoformans*, and PBMC were more potent than adherent cells (Figure 12A). Upon phenotypic examination loosely adherent cells were found to consist of much fewer CD14 positive cells, and were more strongly class II positive (Table 8) than firmly adherent cells. This again raised doubts that the APC for *C. neoformans* was a monocyte, and suggested that it as either a B cell, or a dendritic cell.

4.2.3 Depletion of Cytolytic cells

Previous studies had suggested that monocytes/macrophages were the APC for *C. neoformans*. Since whole *C. neoformans* was used to stimulate T cells it seemed likely a cell with significant cytolytic capabilities would be required to function as the antigen presenting cell. Leucine methyl ester (LME) treatment depletes cytolytic cells. Specifically, it causes lysosomal disruption and selective death of monocytes by concentrating in the lysosome and causing osmotic swelling and eventual rupture (Theile and Lipsky, 1982). By using LME this characteristic could be examined for its contribution to APC function and to help further identify the APC. Monocytes and macrophages are the primary targets of leucine methyl ester treatment; however, it can also deplete natural killer cells, a fraction of TCR $\alpha\beta$ + CD8 + cells and $\gamma\delta$ cells (Theile and Lipsky, 1982; Pechold and Kabelitz, 1993). Treatment with leucine methyl ester has

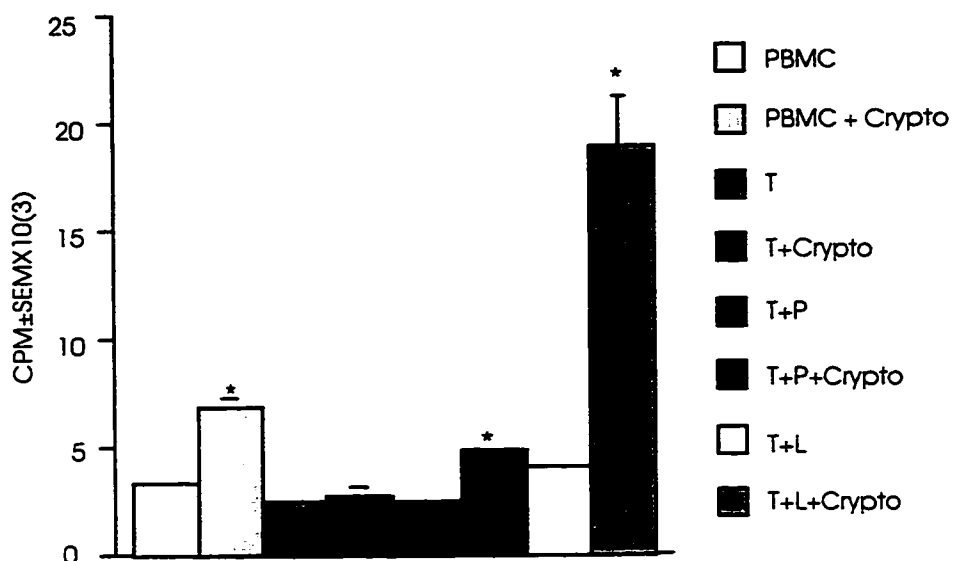


Figure 13: Loosely adherent cells are a potent source of APC for *C. neoformans*. T cells (2×10^5 /well) were stimulated in the presence or absence of *C. neoformans* (2×10^5 /well) with either irradiated PBMC (P; 10^5 /well), or loosely adherent cells (L; 10^5 /well) as a source of APC. Seven days later lymphocyte proliferation was assessed by thymidine incorporation. One of 4 representative experiments.

* $p < 0.05$ as compared to unstimulated control.

little effect on lysosome-poor circulating dendritic cell populations (Theile and Lipsky, 1982). When PBMC were treated with LME the resultant population had less than 1% CD14+ and CD56+ cells. Treating PBMC with LME did not effect their ability to function as APC for T cell responses to *C. neoformans* as determined by lymphocyte proliferation (Figure 14). Additionally, treatment of loosely adherent cells with LME did not abrogate their ability to function as APC (Figure 15). Thus, both these LME treated cell populations were capable of functioning as APC for cryptococcal responses. These results suggest that cytolytic cells are not required to generate a T cell response to *C. neoformans*. As depletion of cytolytic cells has no effect on APC function this also suggests that a monocyte/macrophage is not responsible, but leaves the possibility that the APC may be a B cell or a dendritic cell.

Experiments were then performed to systematically deplete major cell populations in PBMC to determine whether they enriched or depleted the APC. Since loosely adherent cells were the most potent APC examined depletions were carried out on this population. Loosely adherent cells, although enriched for some cell types, may contain other cell populations that contribute to the initiation of T cell responses. It was important therefore to try and further characterize what members of this cell population were contributing to the generation of T cell responses.

4.2.4 Sequential depletion of CD3+, CD14+, CD19+ and CD56+ cells

Cell populations were sequentially depleted from loosely adherent cells by immunomagnetic separation. As each separation was performed, the resultant depleted

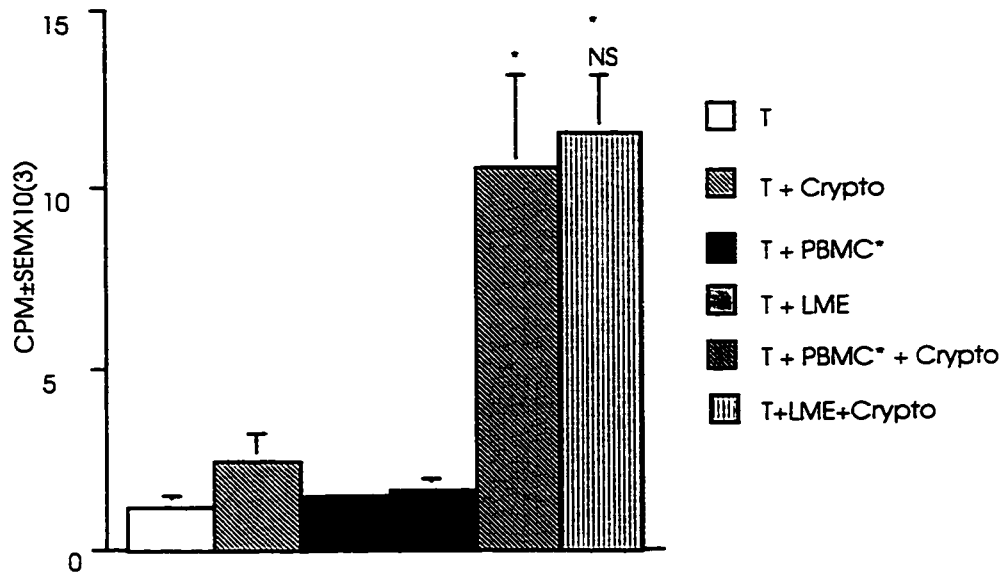


Figure 14: LME treatment of APC has no effect on T cell proliferation to *C.*

neoformans. T cells (2×10^5 /well) were stimulated by *C. neoformans* (2×10^5 /well) in the presence or absence of irradiated PBMC (PBMC*; 1×10^5 /well) or LME treated PBMC (LME; 1×10^5 /well) as a source of APC. Seven days later thymidine incorporation was determined.

* $p < 0.05$ as compared to unstimulated cells. NS- not significant as compared to

T+PBMC*+Crypto. One of three representative experiments.

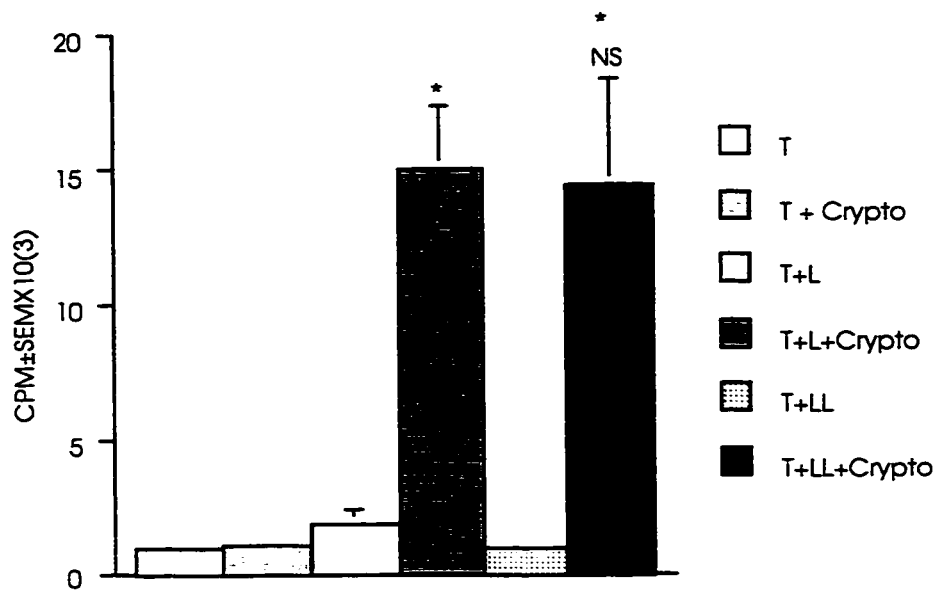


Figure 15: LME treatment of loosely adherent cells has no effect on T cell proliferation to *C. neoformans*. T cells (2×10^5 /well) were stimulated by *C. neoformans* (2×10^5 /well) in the presence or absence of loosely adherent cells (L; 10^5 /well) or LME treated irradiated loosely adherent cells (LL; 10^5 /well). Seven days later thymidine incorporation was determined.

* $p < 0.05$ as compared to unstimulated control. NS- not significant as compared to T+L+Crypto. One of three representative experiments

population was tested for its ability to induce T lymphocyte responses to *C. neoformans*. These depleted cell populations were found to be capable of inducing lymphocyte proliferation, as well as, and sometimes better than the original loosely adherent population (Figure 16 A-D).

CD3+ cells are the most prevalent leukocyte population of cells in peripheral blood, and were unlikely to contribute to antigen processing and presentation. It was not surprising when this population was depleted there was no inhibition of the antigen processing and presentation function of the remaining cells (Figure 16A). This depleted population was more potent than loosely adherent cells alone in inducing T cell responses to *C. neoformans*, which may be related to an enrichment of monocytes, or more likely of B cells or dendritic cells.

CD14+ cells are mostly monocytes, which can be important antigen presenting cells in many systems (Unanue and Allen, 1987). This population was initially believed to be the most likely APC for *C. neoformans*. When these cells (CD14+) and CD3+ cells were depleted the remaining cells were again potent inducers of lymphocyte proliferation to *C. neoformans* (Figure 16B). Further, there was no loss of APC function in the absence of CD14+ cells as compared to cells that were loosely adherent, again suggesting that CD14+ cells were not required for successful processing and presentation of *C. neoformans*.

B cells are potent APC for recall responses (Chestnut and Gray, 1986). Next the CD3 and CD14 depleted loosely adherent cells were depleted of CD19+ cells. This

Figure 16 A-D: Sequential depletion of loosely adherent cells resulted in cell populations that could induce T cell responses to *C. neoformans*.

Panel A) T cells (2×10^5 /well) were stimulated *with C. neoformans* (2×10^5 /well) in the presence of loosely adherent cells (L; 1×10^5 /well), or CD3 depleted cells (CD3-; 1×10^5 /well) as a source of APC.

Panel B) T cells (2×10^5 /well) were stimulated *with C. neoformans* (2×10^5 /well) in the presence of loosely adherent cells (L; 5×10^4 /well), or CD3 and CD14 depleted cells (CD14-/CD3-; 5×10^4) as a source of APC.

Panel C) T cells (2×10^5 /well) were stimulated *with C. neoformans* (2×10^5 /well) in the presence of loosely adherent cells (L; 5×10^4 /well), or CD3, CD14 and CD19 (CD19-/CD14-/CD3-; 5×10^4 /well) depleted cells as a source of APC.

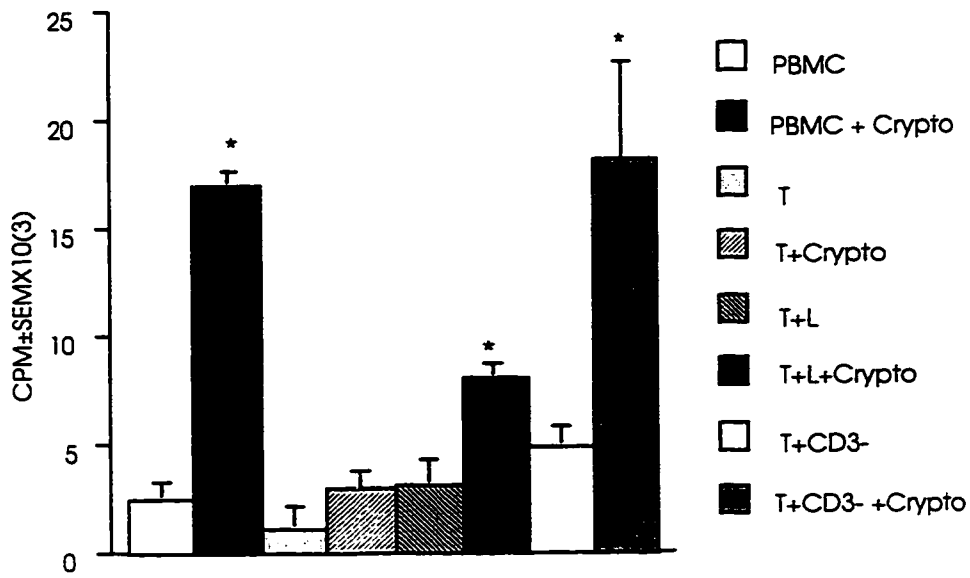
Panel D) T cells (2×10^5 /well) were stimulated *with C. neoformans* (2×10^5 /well) in the presence of loosely adherent cells (L; 2.5×10^4 /well), or CD3, CD14, CD19 and CD56 depleted cells (CD56-/CD19-/CD14-/CD3-; 2.5×10^4 /well) as a source of APC.

Seven days later thymidine incorporation was determined.

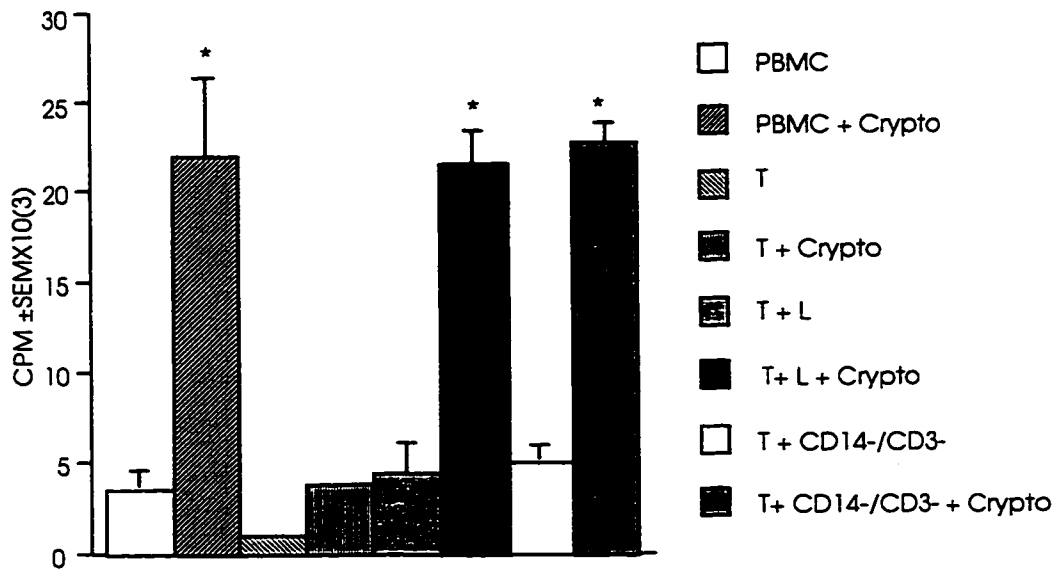
* $p < 0.05$ as compared to appropriate unstimulated control.

Each panel is one of at least three representative experiments.

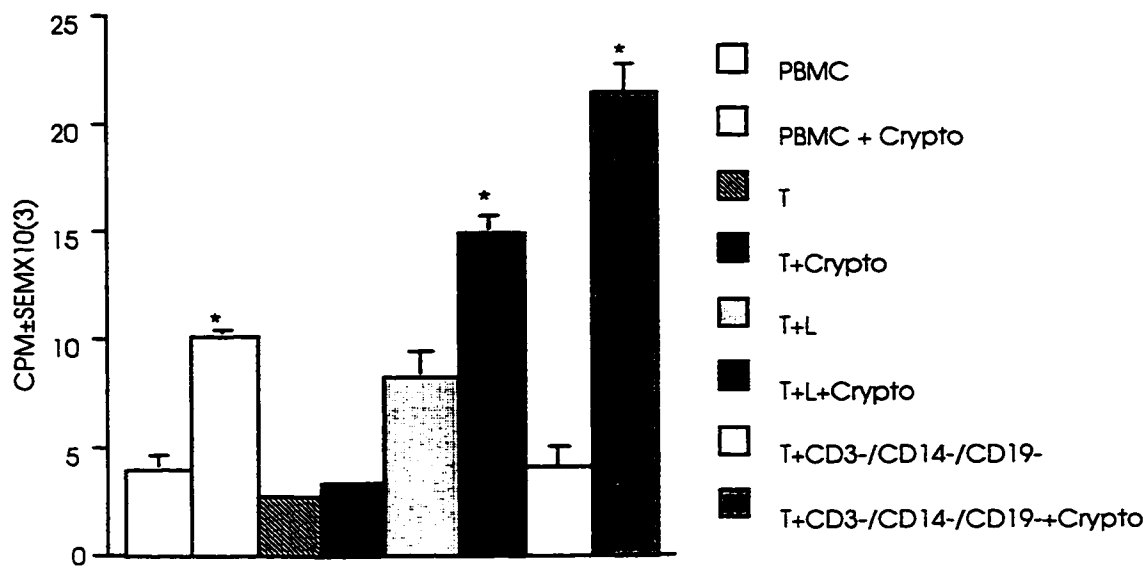
16A



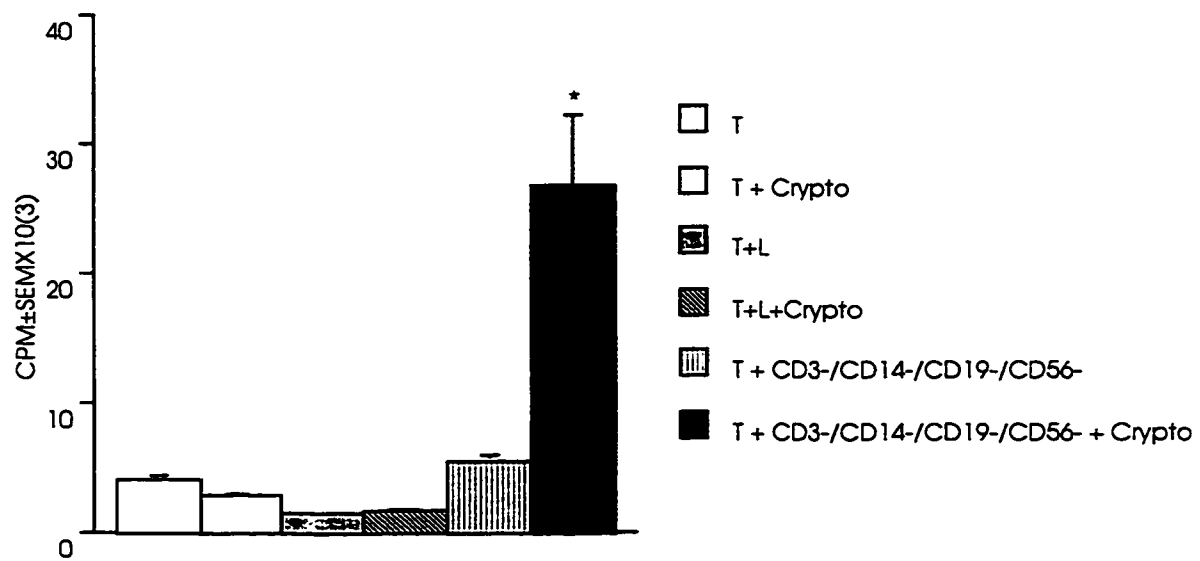
16B



16C



16D



further depletion of CD19+ cells did not effect lymphocyte proliferation. The remaining cells were still as potent as previous populations at inducing lymphocyte proliferation (Figure 16C).

The remaining major cell population in PBMC are natural killer cells. Thus, the population depleted were CD56 positive cells (natural killer cells). Although these cell have never been described as having antigen presenting cell function they do have anti-cryptococcal activity (Murphy and McDaniel, 1982) and may disrupt *C. neoformans* allowing for the presentation by “professional” APC. It should be noted that as different cell populations were depleted it became necessary to drop the APC number (from 1×10^5 to 2.5×10^4) to reduce the autologous mixed lymphocyte reaction that occurred with the remaining cells. At this low cell number loosely adherent cells were often unable to elicit a T cell response (Figure 16D). Depletion of CD56 positive cells did not effect the presentation of *C. neoformans*. The final cell population (CD3, CD14, CD19 and CD56 depleted) was capable of inducing potent T cell responses to *C. neoformans* (Figure 16D).

4.2.5 Characterization of depleted population

When examined by flow cytometry this final depleted cell population was found to express low levels of CD3, CD14, CD19 or CD56, high levels of HLA-DR and CD83 (Table 9), the characteristic phenotype of peripheral blood dendritic cells (Freudenthal and Steinman, 1990; Zhou and Tedder, 1993). Further, if these cells were cultured in RPMI media even more cells expressed HLA-DR and CD83 (Table 9).

Table 9: Phenotype of CD3-/CD14-/CD19-CD56- loosely adherent cells.

<i>Cell Marker</i>	<i>Freshly Isolated CD3-/CD14-/CD19-/CD56- Loosely Adherent Cells (0 hours)</i>	<i>Cultured Isolated CD3-/CD14-/CD19-/CD56- Loosely Adherent Cells (120 hours)</i>
HLA-DR	66.0±5.5	90.0±3.5
CD83	40.1±3.5	71.3±13.0
CD3	2.4±1.2	ND
CD14	1.3±1.8	ND
CD19	2.0±1.0	ND
CD56	2.2±0.9	ND

Mean of 6 experiments.

ND - Not done

consistent with the observations of Zhou and Tedder (1993) and Banchereau and Steinman (1998).

The morphology of the depleted cells was examined. Cells were incubated in glass tissue culture chambers prior to being stained with Diff Quik_R and examined by light microscopy. These cells were found to have characteristic dendritic morphology (Figure 17). Cells were large and possessed an irregular shape. They had large irregular nuclei and lacked a granular cytoplasm. Thin, delicate “dendritic” cytoplasmic processes could be observed extending from the cell body. These processes became more pronounced if the cells had been cultured for a period of time (Ossevoort *et al.*, 1992).

Blood derived dendritic cells are potent inducers of mixed lymphocyte responses especially as compared to unseparated PBMC (Andreesen and Hart, 1993). To ensure that this depleted population functioned in a manner consistent with dendritic cells these cells were tested in a mixed lymphocyte response (Figure 18). When compared to the same low number of irradiated PBMC (12.5×10^3 cells/well) dendritic cells were capable of inducing a very strong mixed lymphocyte response, and were significantly better than the PBMC (Figure 18).

Having fulfilled the following criteria: 1) a dendritic morphology
2) functioning in a mixed lymphocyte response 3) expressing high levels of HLA-DR and CD83 4) not being cytolytic 5) lacking expression of markers for T cells, monocytes, B cells or NK cells, the CD3-/CD14-CD19-/CD56- loosely adherent cells will now be referred to as dendritic cells.

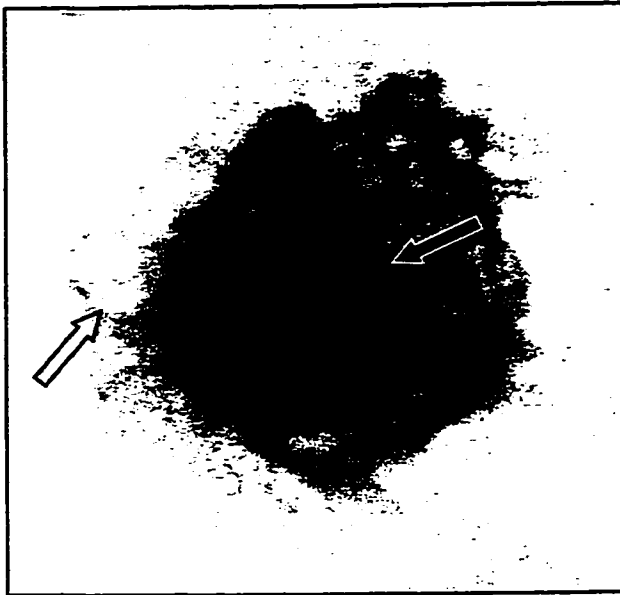


Figure 17: CD3, CD14, CD19 and CD56 depleted cells have dendritic morphology.

Loosely adherent cells were depleted of CD3, CD14, CD19 and CD56 positive cells. 5×10^5

cells were placed in glass tissue culture chambers, and 18 hours later the slides were air dried, stained with Diff Quik_R and examined by light microscopy.

Open arrows indicate dendritic processes. Closed arrows indicates large irregular nucleus.

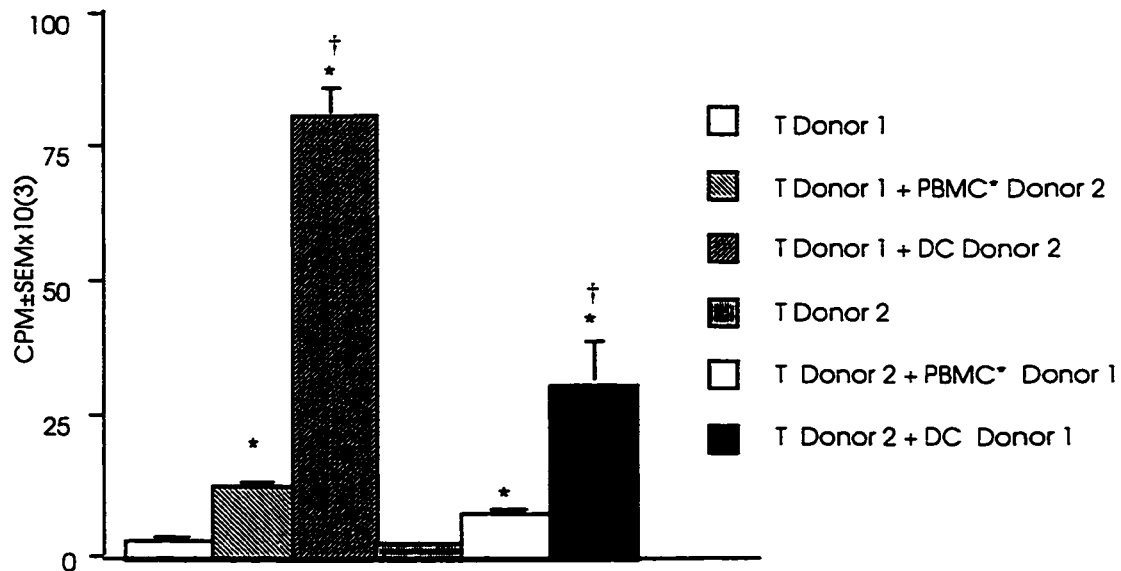


Figure 18: CD3, CD14, CD19 and CD56 depleted cells can induce potent mixed lymphocyte responses.

T cells and dendritic cells were purified from two donors. T cells (2×10^5 /well) from one donor were put into culture with irradiated PBMC (12.5×10^3 /well), or dendritic cells (12.5×10^4 /well) from the second donor. Lymphocyte proliferation was assessed by thymidine incorporation 5 days later. One of four representative experiments.

* $p < 0.05$ as compared to T cells alone

† $p < 0.05$ as compared to T cells + PBMC

4.2.6 Number of dendritic cells required to induce T cell responses

Having established that dendritic cells could induce lymphocyte proliferation this population was assessed to determine the number of dendritic cells required to induce an antigenic T cell response. When a dose response was investigated it was found that a very limited number of dendritic cells was capable of producing a significant lymphocyte proliferative response to *C. neoformans* (Figure 19). A strong autologous mixed lymphocyte reaction appeared until the antigen presenting cell number was decreased to 2.5×10^4 cells/well. This was much lower than the number of loosely adherent cells required to produce the same response (Figure 19). A four fold reduction in the number of dendritic cells produced a similar proliferative response to loosely adherent cells. Subsequent experiments were carried out to see whether the number of dendritic cells required to induce a T cell response to *C. neoformans* could be lowered any further. As few as 3125 dendritic cells were capable of inducing a significant T cell response (Figure 20). This was consistent with previously described responses evoked by dendritic cells (VanVoorhis *et al.*, 1983).

4.2.7 Microscopic examination of dendritic cells and *C. neoformans*

Light Microscopy

The mechanism of the interaction between dendritic cells and *C. neoformans* was investigated. Historically, studies have indicated that dendritic cells are not phagocytic (Steinman and Cohn, 1974), but could take up various particulates of $< 3 \mu\text{m}$ in size (Inaba *et al.*, 1993). Although dendritic cells are known to have the capacity to be

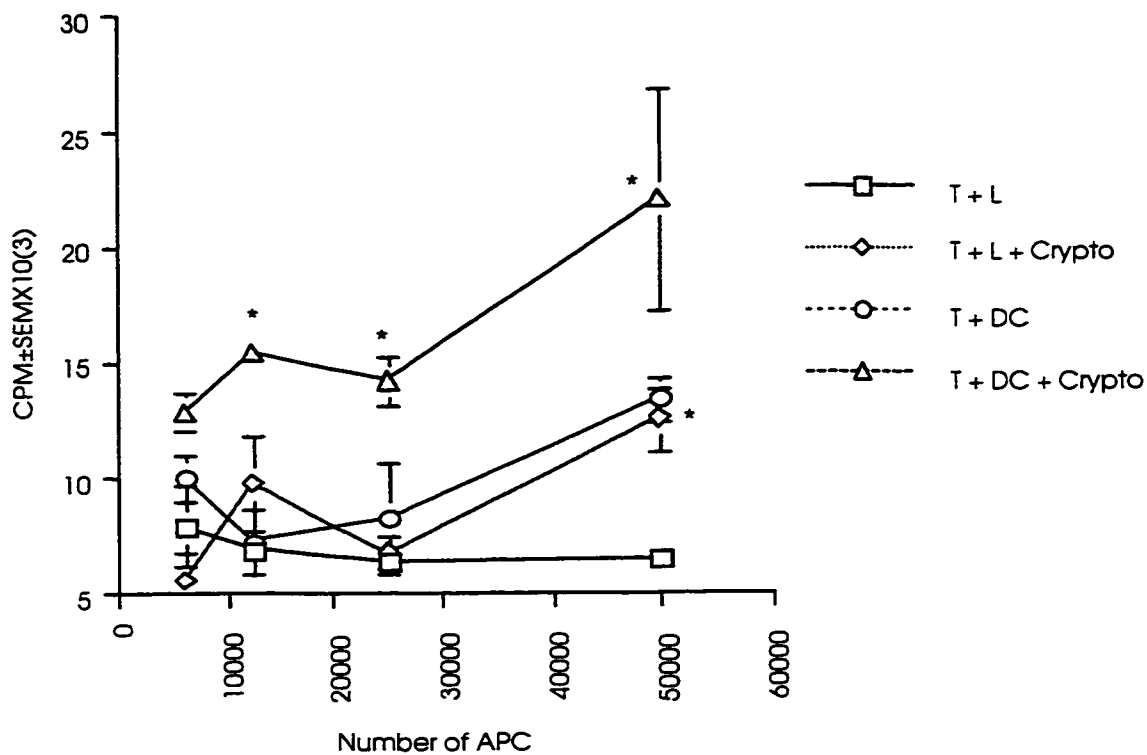


Figure 19: Dendritic cells are more potent APC than loosely adherent cells at a low cell number. Purified T cells (2×10^5 /well) were put into culture with *C. neoformans* (2×10^5 /well) and varying numbers of loosely adherent (L) or dendritic cells (DC). Lymphocyte proliferation was assessed 7 days later. One of three representative experiments.

* $p < 0.05$ as compared to corresponding control.

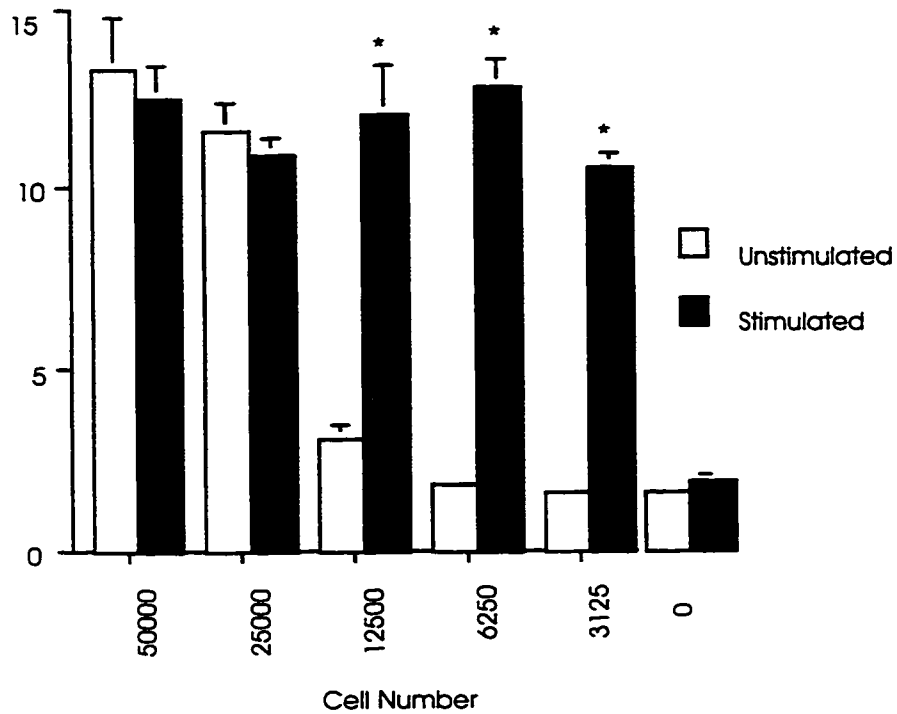


Figure 20: Dose response of the number of dendritic cells required to induce T cell proliferation to *C. neoformans*. T cells (2×10^5 /well) were put into culture with various numbers of dendritic cells in the presence or absence of *C. neoformans* (2×10^5 /well). Lymphocyte proliferation was assessed 7 days later by thymidine incorporation. * $p < 0.05$ as compared to appropriate unstimulated control. The experiment was repeated with similar results.

endocytic (Levine and Chain, 1992; Reis e Sousa *et al.*, 1993; Steinman and Swanson, 1995) there are only limited data implicating a dendritic cell as important antigen presenting cell for microbial pathogens (Filgueria *et al.*, 1996; Mbow *et al.*, 1997). Further, large microorganisms ($>3 \mu\text{m}$), such as *C. neoformans* (Levitz, 1991a), have never been described being taken up and presented to T cells by dendritic cells, and no fungal pathogen has been reported to be taken up by dendritic cells, in any system. It is possible that dendritic cells may degrade antigen at the cell surface (extracellularly), a suggestion put forward by Steinman and Swanson (1995). It is also a possibility that these cells acquire peptides from other cells for presentation (Kaye *et al.*, 1985). Initially, cells were examined by light microscopy to assess their ability to internalize *C.*

neoformans. Dendritic cells were incubated with *C. neoformans* for 18 hours at 37°C on glass chamber slides. At the end of incubation cells were fixed and stained with Giemsa for examination under light microscopy. *C. neoformans* was considered internalized if they were completely surrounded by dendritic cell membrane and a vacuole could be seen surrounding the organism. Surprisingly, dendritic cells could both bind and take up this large pathogen (Figure 21). Dendritic cells were observed with completely internalized *C. neoformans* with vacuoles surrounding the ingested organisms.

Scanning Electron Microscopy

To get more detail as well as a greater appreciation of the interaction between dendritic cells and *C. neoformans*, cells were examined by scanning electron microscopy (generously performed by Dr. F. Green). Cell cultures were carried out as for light

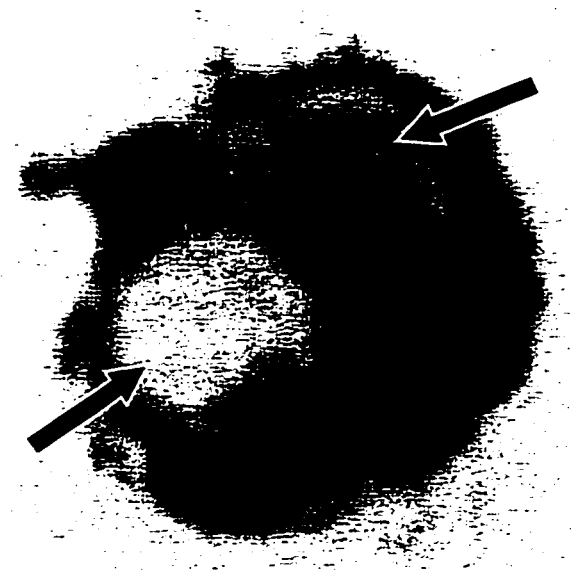


Figure 21: *C. neoformans* is internalized by dendritic cells as shown by light microscopy. Purified dendritic cells were put into culture *with C. neoformans* for 18 hours. Cells were then washed, fixed, stained with Giemsa and examined by light microscopy. Arrows indicate internalized *C. neoformans*.

microscopy. This examination demonstrated that dendritic cells were capable of interacting with multiple organisms both on the cell surface body as well as by interactions with the dendrites. The long processes radiating from the cell body bound multiple organisms. It again appeared as if the organisms were within the dendritic cell suggesting that the cells were capable of phagocytosing the organisms as demonstrated by the contours of the cell (Figure 22).

Transmission Electron Microscopy

As it has very rarely been reported that human dendritic cells can take up bacteria (Mbow et al 1997; Filgueria *et al.*, 1996) and has never been reported for fungi or parasites, it was important to ensure that the cryptococcal organisms were indeed internalized. Examination by transmission electron microscopy (generously performed by Leona Barclay) (Figure 23B) confirmed that the organisms were indeed internalized by dendritic cells. Further, these cells had similar features to peripheral blood dendritic cells described in the literature (Knight *et al.*, 1992; Zhou and Tedder, 1995). The mononuclear cells possessed large oval or irregularly shaped nuclei, many mitochondria, few lysosomes, and numerous vesicles, and short dendritic arms coming off all parts of the cell body that appeared to “reach” toward the organism (Figure 23A). These blunt projections become more veiled in appearance as the cells mature (Knight *et al.*, 1992). Dendritic cells with internalized *C. neoformans* were observed, and a thin vacuolar membrane could be observed surrounding the organisms (Figure 23B).

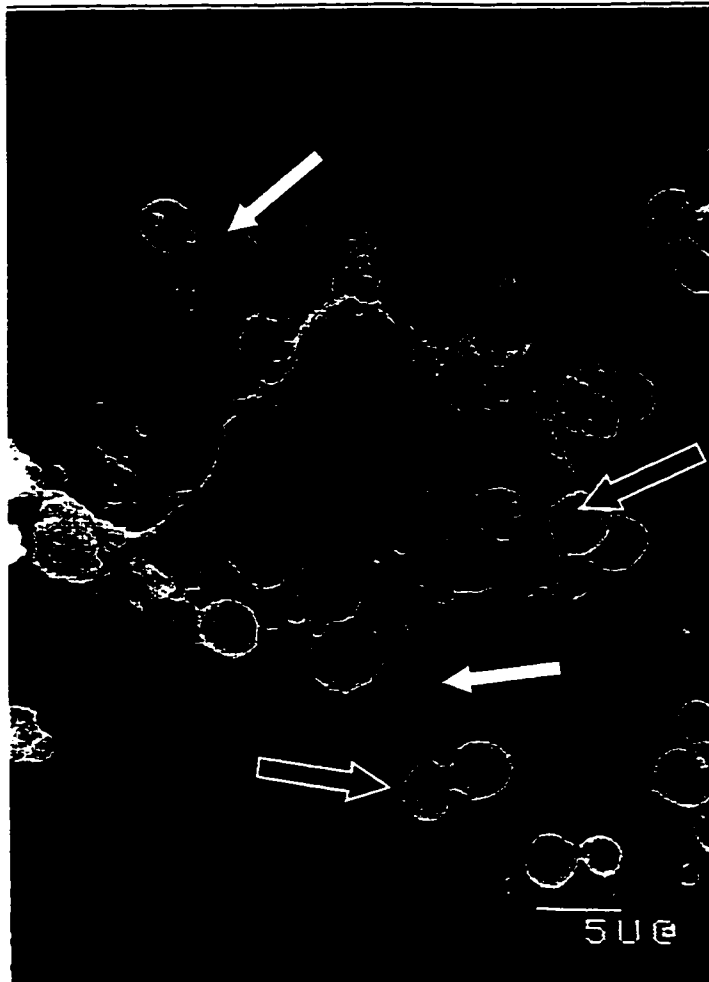


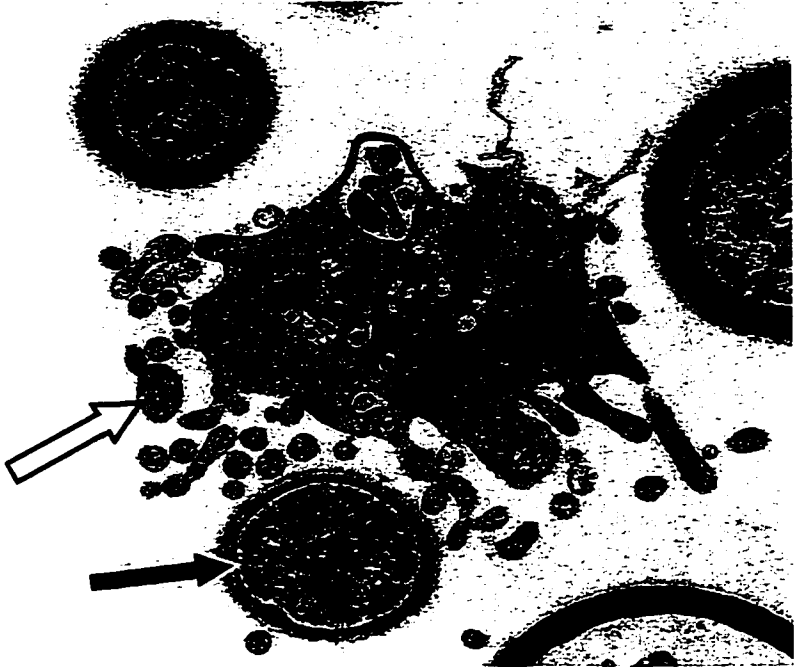
Figure 22: *Cryptococci* bind to dendritic cells as shown by scanning electron microscopy. Dendritic cells were put into culture at a 1:10 ratio with *C. neoformans*. 18 hours later cells were spun fixed in 2.5% glutaraldehyde 0.1 M sodium cacodylate buffer and examined by scanning electron microscopy. White arrows indicate dendritic processes.

Black arrows indicate *C. neoformans*.

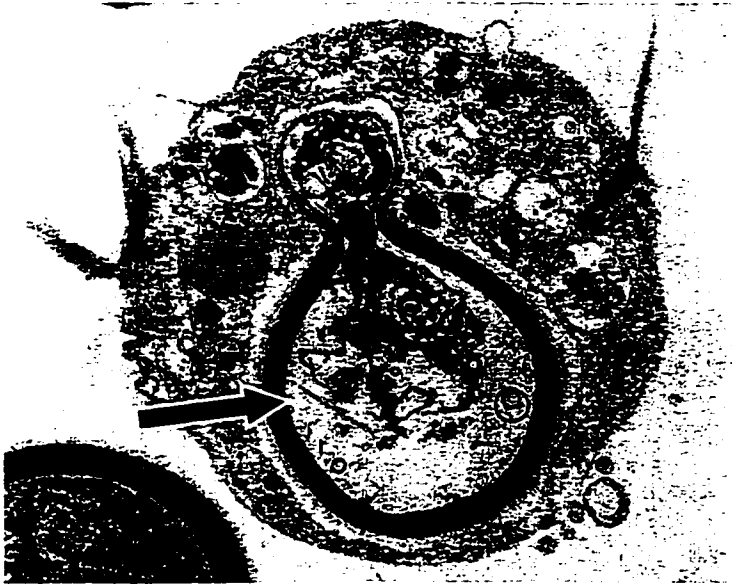
Figure 23A: Dendritic cells and *C. neoformans* as shown by transmission electron microscopy. Dendritic cells were put into culture at a 1:10 ratio with *C. neoformans*. 18 hours later cells were spun fixed in 2.5% glutaraldehyde 0.1M sodium cacodylate buffer and examined by transmission electron microscopy (X 8000). White arrow indicates dendritic arms. Black arrow indicates *C. neoformans*.

Figure 23B: *C. neoformans* are internalized by dendritic cells as shown by transmission electron microscopy. Dendritic cells were put into culture at a 1:10 ratio with *C. neoformans*. 18 hours later cells were spun fixed in 2.5% glutaraldehyde 0.1M sodium cacodylate buffer and examined by transmission electron microscopy (X8000). Black arrow indicates *C. neoformans*.

23A



23B



4.2.8 Quantitation of uptake by dendritic cells

A variety of hypotheses have been put forth to reconcile the poor endocytic ability of dendritic cells and their immunostimulatory capacity (Steinman and Swanson, 1995), including a low endocytic requirement. To determine how endocytic dendritic cells were, their ability to bind and internalize *C. neoformans* was quantitated. Different complimentary techniques were used. Giemsa staining allowed for individual cells to be examined. By Giemsa staining the cells could be carefully examined for dendritic morphology, and the number of internalized and bound organisms could be delineated. Further, this allowed assessment of binding and uptake without further manipulation of the system such as labeling the organisms with FITC and flow cytometric analysis. FITC-conjugation and flow cytometric analysis had the advantage of allowing many more cells to be examined. Further, intracellular and extracellular organism could be differentiated by quenching, which eliminated florescence of extracellular organisms.

Dendritic cells were put into culture with *C. neoformans*, eighteen hours later cytopspins were prepared and the cells stained with Giemsa and examined by light microscopy as has been described for *C. neoformans* and monocytes (Kozel, 1977). *C. neoformans* was considered to be bound to a dendritic cell if a dendritic processes could be observed interacting with the microbe, but this interaction did not completely encompass the organism or there was no evidence of a vacuolar membrane around the cell. By this technique $42.3 \pm 10.3\%$ of dendritic cells bound cryptococcal organisms with a range of 0-7 organisms binding per dendritic cell (Figure 24). Between 15 and 34 %

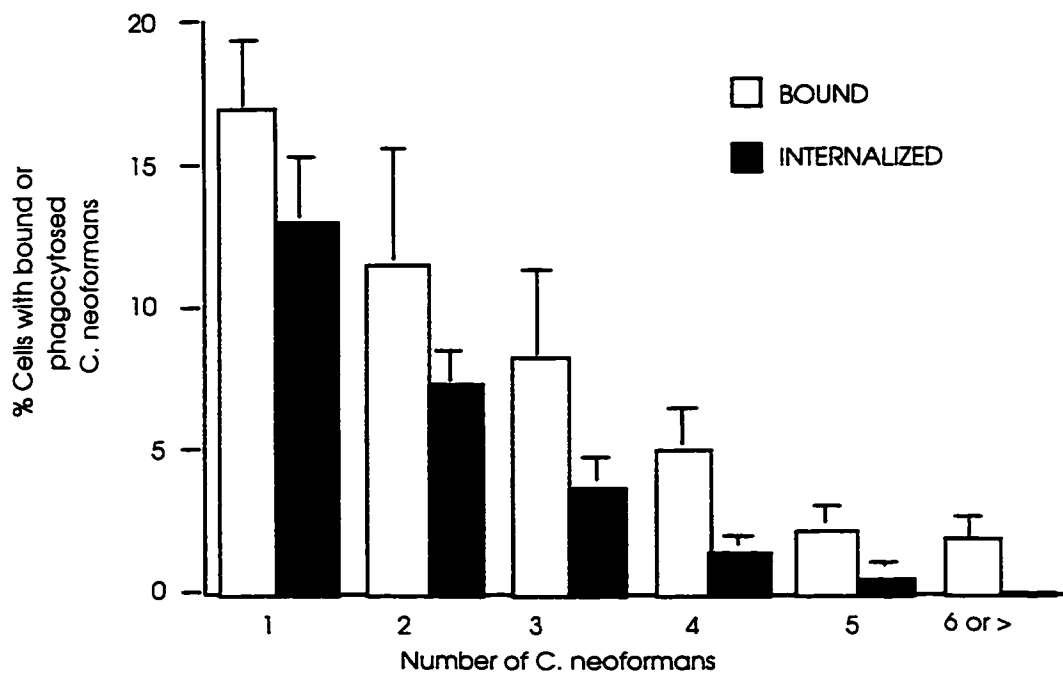


Figure 24: Number of dendritic cells that bind and internalize *C. neoformans*.

Dendritic cells (5×10^5) were put into culture with *C. neoformans* (5×10^6) and examined 18 hours by giemsa staining and light microscopy. At least 200 cells were examined per experiment.

Mean of 4 experiments.

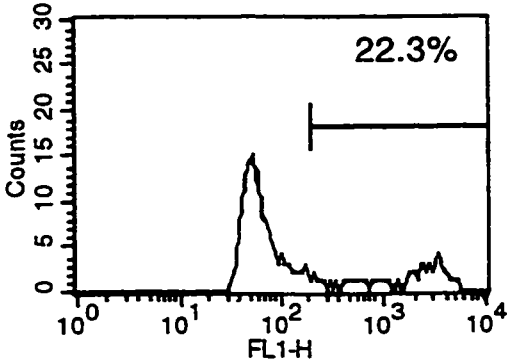
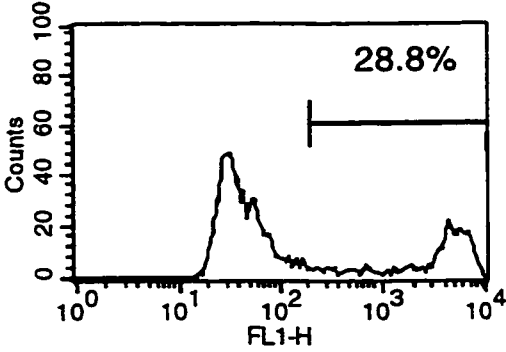
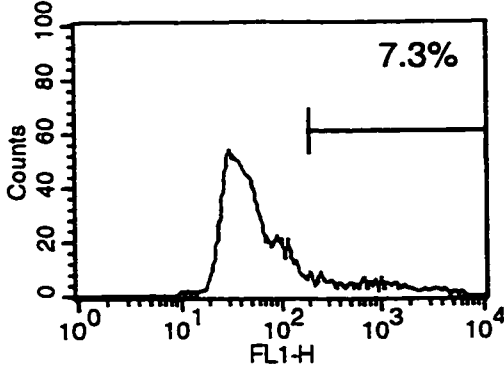
(with an average of $24.9 \pm 3.1\%$) of dendritic cells had taken up *C. neoformans* (Figure 24). Individual cells were observed taking up as many as 6 organisms.

Uptake was also assessed by using FACS analysis as per Chaka and colleagues (1995). Briefly, cells were incubated with FITC-labeled organisms and prior to analysis, cells were incubated with trypan blue to quench the extracellular fluorescence. This allowed the external or bound organisms to appear blue and left the internalized organisms to continue to fluoresce. When assessed by FACS analysis this technique revealed a similar number of internalized organisms as analysis by Giemsa staining (Figure 25). The limit of detection of this assay was determined by sorting cells on a flow cytometer into FITC positive and negative populations. The negative population contained no *Cryptococcus* associated dendritic cells, while the positive cells had at least one internalized organism. The low percentage of bound organisms may reflect of a loss of this population by the nature of the flow cytometric procedure which may dislodge the organisms. It is also possible that bound organisms were over represented by light microscopic examination.

4.2.9 Role of mannose receptor in the interaction between dendritic cells and *C. neoformans*

Having determined that dendritic cells were important antigen presenting cells for T cell responses to *C. neoformans* experiments were performed to determine how this cell interacted with the microbe. The surface characteristics of *C. neoformans* and the high

Figure 25: Percentage of dendritic cells that internalize *C. neoformans* as shown by flow cytometry. Dendritic cells (1×10^6) were put into culture with FITC- labeled *C. neoformans* (10×10^6). 18 hours later cells were examined by FACS analysis prior to and after extracellular fluorescence was quenched with trypan blue. At least 10 000 events were examined per group. Panel A - Dendritic cells alone, Panel B - Dendritic cells + FITC conjugated *C. neoformans*, Panel - C Dendritic cells + FITC conjugated *C. neoformans* after trypan blue quenching. One of 3 representative experiments.



levels of expression of the mannose receptor on dendritic cells (Ezekowitz *et al.*, 1990) made the mannose receptor a likely candidate for the mechanism by which *C. neoformans* is bound and taken up by dendritic cells. Mannan has been demonstrated to bind to the mannose receptor ligand and prevent ingestion via this receptor (Engering *et al.*, 1997; Prigozy *et al.*, 1997). APC were incubated with *C. neoformans* in the presence of mannan or mannose (which does not bind the mannose receptor; Prigozy *et al.*, 1997) as a control ligand. Addition of mannan reduced dendritic cell dependent lymphocyte proliferation (Figure 26A). The addition of mannan to the culture reduced lymphocyte proliferation by 52%. As a control, the response to SEB was assessed. Mannan had no effect on responses to SEB, which would not interact with this receptor (Figure 26B).

A second approach was used to demonstrate the role of the mannose receptor in the proliferative response. An antibody directed to the mannose receptor could abrogate T cell responses to *C. neoformans* (Figure 27). There was a 68% reduction in the response. This suggests that the mannose receptor is required for binding that resulted in antigen presentation of *C. neoformans*, but does not eliminate the possibility that other receptors may also be involved in interactions between dendritic cells and *C. neoformans*.

4.2.10 Organ specific responses

Although processing of *C. neoformans* is likely to occur within tissue the initial contact of *C. neoformans* with the immune system occurs within the airspaces of the lung. Experiments were performed to determine if *C. neoformans* could be processed by cells obtained from the lung. It may be that *C. neoformans* is actively phagocytosed in one

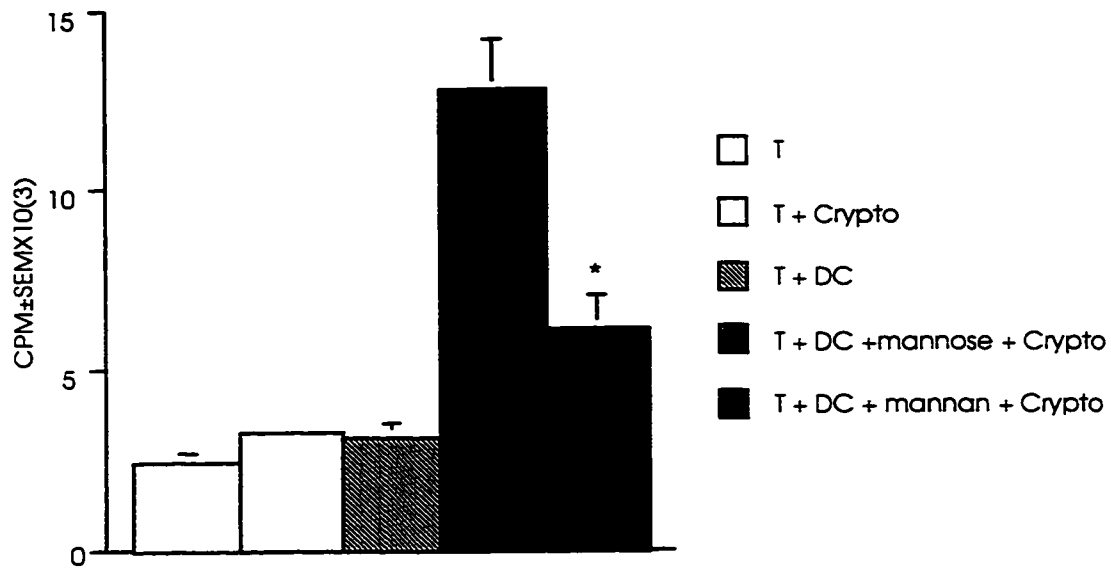
Figure 26 A: Blocking the mannose receptor with mannan inhibits T lymphocyte proliferation to *C. neoformans*. T cells (2×10^5 /well) were put into culture with dendritic cells (DC; 2.5×10^4 /well) and *C. neoformans* (2×10^5 /well) in the presence or absence of $3 \mu\text{g/ml}$ mannan or mannose as a control. Seven days later lymphocyte proliferation was assessed by thymidine incorporation. One of four representative experiments.

* $p < 0.05$ as compared to T+DC+Crypto+mannose

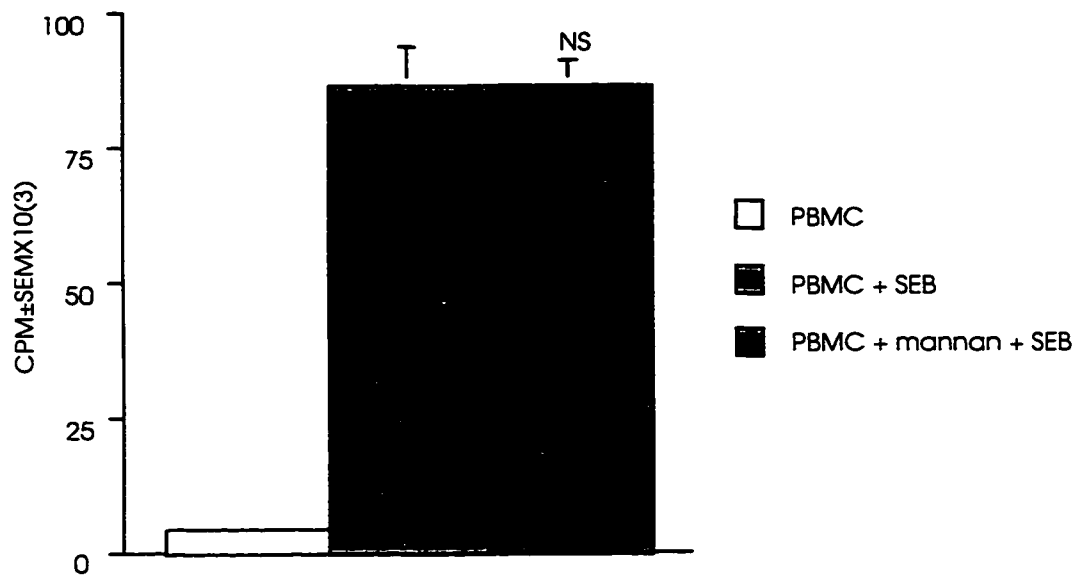
Figure 26B: Mannan does not block the proliferative response to the superantigen SEB. PBMC (2×10^5 /well) were put into culture with SEB ($1 \mu\text{g/ml}$) in the presence or absence of $3 \mu\text{g/ml}$ of mannan. Five days later lymphocyte proliferation was assessed by thymidine incorporation. One of two experiments.

NS - not significantly different from PBMC + SEB

26A



26B



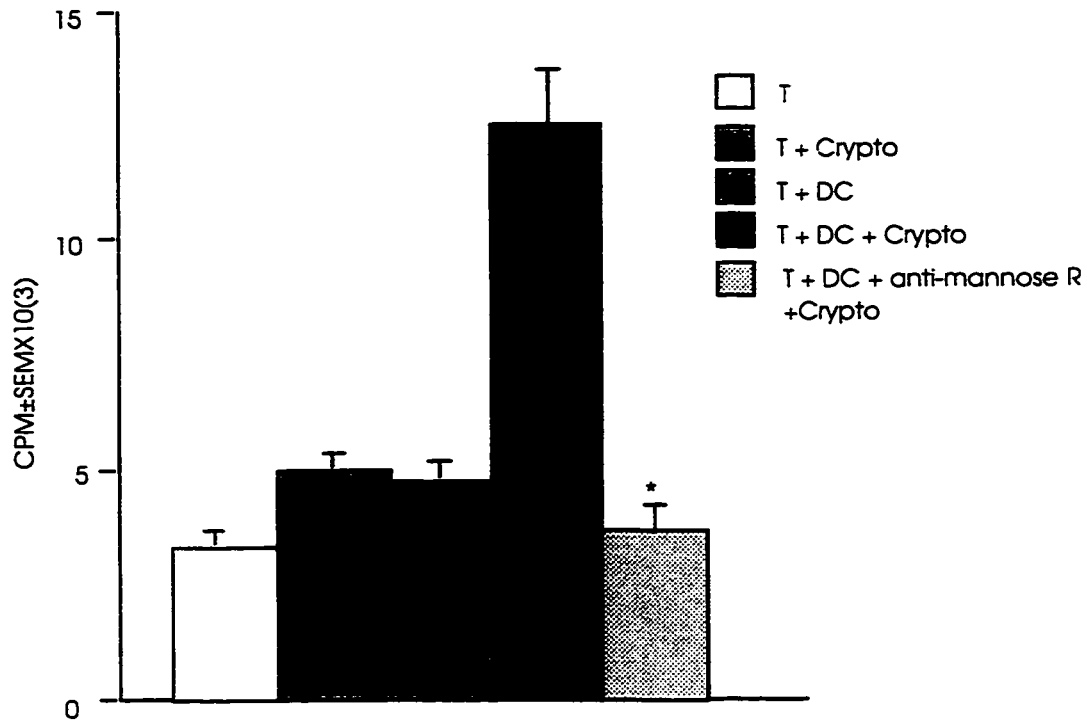


Figure 27: Anti-mannose receptor antibody blocks T lymphocyte proliferation to *C. neoformans*. T cells (2×10^5 /well) were put into culture with dendritic cells (DC; 2.5×10^4 /well) and *C. neoformans* (2×10^5 /well) in the presence or absence of $10 \mu\text{g/ml}$ anti-mannose receptor antibody. Seven days later lymphocyte proliferation was assessed by thymidine incorporation. One of three representative experiments.

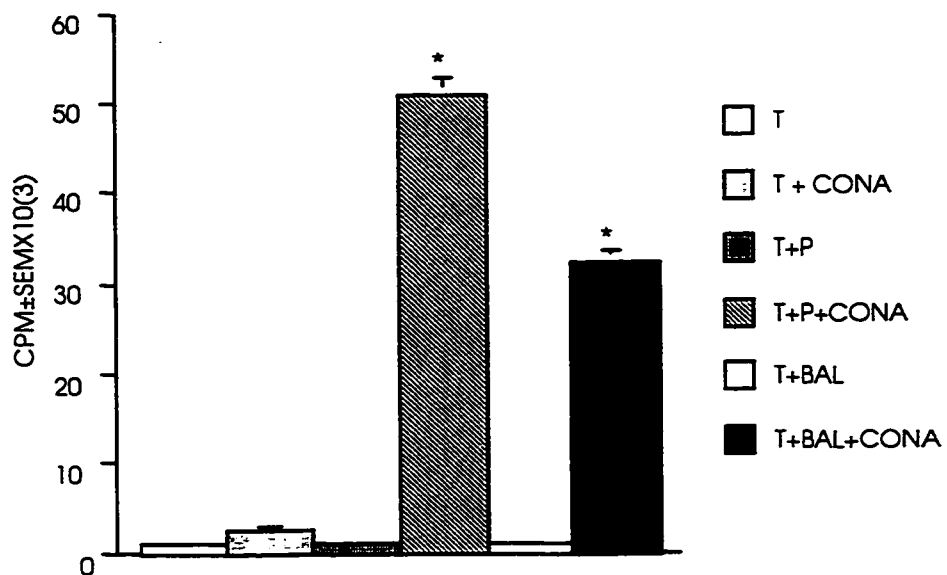
area (lung) and then processed and presented in another by migrating to a regional lymph node or the spleen. Possible antigen presenting cells obtained from BAL consist of a large number of alveolar macrophages (~95%), a small number of monocyte like cells, and a minor population of dendritic cells (Van Haarst *et al.*, 1994). The typical number of dendritic cells in BAL is 0.3-1.6%, which have been suggested to be the major accessory cell in BAL (Havenith *et al.*, 1994), as alveolar macrophages are considered immunosuppressive (McCombs *et al.*, 1982; Toews *et al.*, 1984). Further, dendritic cell enrichment of BAL cells significantly augments T cell responses (Havenith *et al.*, 1989). To determine whether local antigen presenting cells could induce T cell responses, cells were isolated from bronchoalveolar lavage from healthy individuals who were undergoing bronchoscopy for minimal hemoptosis. Samples were collected into saline. Cells were then filtered and washed to remove mucous.

Individuals on whom BAL was performed had normal proliferative responses to a T cell mitogen, Con A (Figure 28A). BAL cells, however, were less effective as a source of APC than irradiated PBMC. When BAL cells were used as a source of APC they caused significant T cell proliferation to *C. neoformans*, but in most cases were not as potent as loosely adherent cells (Figure 28B). It was found that cells obtained by BAL could indeed present cryptococci and induce T cells responses in healthy patients (Figure 27B). The cell population within BAL that is performing this APC function has not yet been determined.

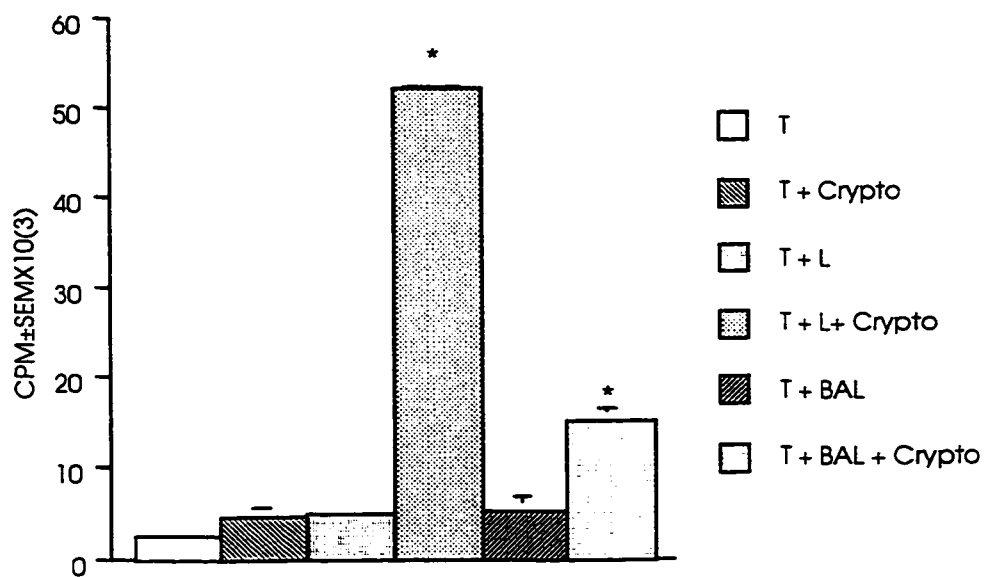
Figure 28A: Cells from bronchoalveolar lavage can induce T cell responses to Con A. T cells (2×10^5 /well) were incubated in the presence of irradiated PBMC (P; 10^5 /well) or irradiated BAL cells (BAL; 10^5 /well) in the presence or absence of Con A ($2.5 \mu\text{g/ml}$). Three days later lymphocyte proliferation was assessed by thymidine incorporation. One of three representative experiments. $*p < 0.05$ as compared to unstimulated control.

Figure 28B: Cells from bronchoalveolar lavage can induce T cell responses to *C. neoformans*. T cells were incubated in the presence loosely adherent cells (L; 10^5 /well) or irradiated BAL (BAL; 10^5 /well) cells in the presence or absence of *C. neoformans*. Seven days later lymphocyte proliferation was assessed by thymidine incorporation. One of five representative experiments. $*p < 0.05$ as compared to unstimulated control.

28A



28B



4.3 DISCUSSION

4.3.1 Antigen Presenting Cell

An absolute requirement for cell mediated immunity is antigen processing and presentation. To be presented, *C. neoformans* has to bind an APC, be taken up, enter endocytic compartments and be degraded to prepare cryptococcal peptides for binding to MHC. It has been assumed up to now that the most likely candidate for such robust requirements would be a macrophage (Collins and Bancroft, 1991). Initial experiments determined that T cells required an antigen processing cell if they were to respond to *C. neoformans*. Subsequently, the nature of this antigen presenting cell requirement for presentation of *C. neoformans* was defined.

By investigating APC it was found that the APC had a number of unique characteristics. 1) the cell was loosely adherent, LME resistant and CD3-/CD14-/CD19-/CD56-/DR+ and CD83+ 2) it had morphologic features of a dendritic cell.

These studies established that the APC for *C. neoformans* was not a cytolytic cell, expressed high levels of HLA-DR and was loosely adherent to plastic, a technique that depleted CD14+ monocytes and enriched for dendritic cells in other systems (Steinman and Cohn, 1974; Van Voorhis *et al.*, 1982; Nicod *et al.*, 1987). Subsequent depletion studies confirmed that this important APC was a dendritic cell. As there are no definitive dendritic cell markers, the purified cells were assessed phenotypically, morphologically and functionally. These data showed that 1) sufficient quantities of purified dendritic cells could be obtained to perform experiments 2) these cells could induce T cell

responses to whole *C. neoformans* 3) these cells could bind and internalize the organism
4) the mannose receptor was an important component of antigen presentation of *C. neoformans*.

Dendritic cells are one of the most potent antigen presenting cells of the immune system. Although their phylogeny is still under debate, it is known that these cells can arise from the bone marrow or peripheral blood as dendritic cell precursors, or develop from CD14+ blood monocytes (Reid *et al.*, 1992; Young *et al.*, 1995; Cella *et al.*, 1997). These precursors enter the blood stream as immature dendritic cells. Immature dendritic cells are capable of antigen capture, but have a diminished ability to initiate T cell responses (Inaba *et al.*, 1993; Sallusto and Lanzavecchia, 1994). As these cells mature, their phenotype changes and they become more potent at initiating T cell responses, but less proficient at taking up antigen (Sallusto and Lanzavecchia, 1994).

Although dendritic cells are potent APC and have the capacity to be endocytic (Levine and Chain, 1992; Reis e Sousa *et al.*, 1993; Steinman and Swanson, 1995); there are only limited data implicating dendritic cell as important antigen presenting cell for microbial pathogens (Filgueria *et al.*, 1996; Mbow *et al.*, 1997). Previous studies have shown that murine dendritic cells can take up a number of bacterial pathogens including *Escherichia Coli*, *Salmonella typhimurium* (Svensson *et al.*, 1997), *Listeria monocytogenes* (Guzman *et al.*, 1995), *Mycobacterium tuberculosis* (Inaba *et al.*, 1993), and *Bordatella bronchiseptica* (Guzman *et al.*, 1994). Human cytokine-induced dendritic cells can take up *Mycobacterium tuberculosis* (Henderson *et al.*, 1997), but the ability to present *M. tuberculosis* antigens was not demonstrated.

Only a handful of studies have demonstrated that dendritic cells can process bacteria for the induction of T cell responses. Murine langerhans cells have been demonstrated to transport viable *Leishmania* parasites from infected skin to draining lymph nodes to activate antigen specific T cells, and initiate a cell mediated response (Moll *et al.*, 1995). GM-CSF-stimulated bone marrow-derived murine dendritic cells can process live intact gram-negative bacteria for peptide presentation by MHC-I and MHC-II (Svensson *et al.*, 1997). Murine splenic dendritic cells pulsed with live *Borrelia burgdorferi*, when transferred into naive mice, help to mediate protection to a subsequent challenge (Mbow *et al.*, 1997). Dendritic cells from human skin, and monocyte derived dendritic cells have been shown to engulf *B. burgdorferi*, and were able to induce proliferation of *Borrelia* specific T cells (Filgueira *et al.*, 1996). Thus, dendritic cells have the capacity to engulf microorganisms for the induction of T cell responses, although uptake and presentation of fungi, like *C. neoformans*, has not previously been demonstrated.

Most of the work on human dendritic cells has utilized cultured or cytokine induced cells. In these studies monocytes are cultured in the presence of human recombinant GM-CSF and human recombinant IL-4 to generate dendritic cells (Romani *et al.*, 1994; Sallusto and Lanzavecchia, 1994). Although this technique has the advantage of producing large numbers of dendritic cells, it results in a homogenous population of cells, all with an immature phenotype. It is unknown whether this type of ontogeny occurs *in vivo*. By examining freshly isolated dendritic cells instead one is able to look at the dendritic cell population actually present *in vivo*, not one that is artificially

induced. This population of freshly isolated dendritic cells would consist of recent migrants from the bone marrow and a minor population that have undergone maturation (O'Doherty *et al.*, 1994).

The findings presented here provide evidence that primary dendritic cells can take up, and ultimately initiate T cell responses to *C. neoformans*. Primary blood derived dendritic cells have never been demonstrated to take up and present a microbial pathogen/antigen to T cells. These freshly isolated cells were found to be very potent inducers of T cell responses to the microbe. Although dendritic cells are being recognized as being much more phagocytic than initially thought (Levine and Chain, 1992; Sallusto and Lanzavecchia, 1994; Reis e Sousa *et al.*, 1995; Sallusto *et al.*, 1995), and have functions and abilities that change as the cell matures (Sallusto and Lanzavecchia, 1994; Sallusto *et al.*, 1995) no reports have described these cells handling such large microbes (>3 μm in diameter) for the initiation of T cells responses. This is also the first description of a pathogenic fungi being taken up by a dendritic cell. The ability to phagocytose large microbes, like *C. neoformans*, gives dendritic cells even broader endocytic ability than has ever previously been demonstrated. Identification of dendritic cells as the APC for *C. neoformans* also suggests that there is a broader division of labour between those cells involved in anti-cryptococcal activity, and those important in initiating T cell responses. That is a monocyte/macrophage is not required to fulfill both functions.

Dendritic cells have also been shown to acquire a mature immunostimulatory phenotype after culture in TNF- α and GM-CSF (Caux *et al.*, 1992; Sallusto and Lanzavecchia, 1994; Bancherau and Steinman, 1998). Both of these cytokines have been

shown to be induced by *Cryptococcus* (Huffnagle *et al.*, 1996; Chaka *et al.*, 1997; Murphy *et al.*, 1997). This cytokine response may help to mature these cells. Dendritic cells become aggravated after infection with *Mycobacterium tuberculosis* and increase surface expression of costimulatory molecules such as CD54, CD40, B7.1 and MHC I (Henderson *et al.*, 1997). Acapsular *C. neoformans* can enhance expression of B7-1 and B7-2 on monocytes (Vecchiarelli *et al.*, 1998a). It would not seem unreasonable then that *C. neoformans* would have similar effects on dendritic cells.

4.3.2 Mannose Receptor

Dendritic cells have been reported to have two distinct pathways for the uptake of antigen. These include fluid-phase uptake through macropinocytosis and receptor mediated endocytosis for the uptake of exogenous antigens (Lanzavecchia, 1996; Tan *et al.*, 1997). Macropinocytosis is a form of endocytosis that accompanies cell ruffling, and allows for nonselective endocytosis of solute macromolecules (Swanson and Watts, 1995). It is a cytoskeletal dependent process, and has been described mostly in the uptake of macrosolutes. In dendritic cells this process is constitutive. This mechanism has only ever been implicated in the uptake of one microbe, *Salmonella typhimurium* (Alpuche-Aranda *et al.*, 1994).

A number of receptors could be responsible for binding, uptake and initiation of T cell responses to *C. neoformans*. Receptor mediated uptake mechanisms include those via, complement, Fc and mannose receptors (Brown, 1995). CR and FcR are receptors that recognize host proteins bound to the invading organism, while mannose receptors recognize mannosylated antigens of the pathogen itself. Uptake mechanisms for whole

microbes by dendritic cells have been documented. *Bordetella bronchiseptica* is taken up via glycosylated receptors (Guzman *et al.*, 1994), and *Borrelia burgdorferi* is taken up using coiling phagocytosis (Filgueira *et al.*, 1996). Microbial attachment via the mannose receptor on dendritic cells for processing and presentation to T cells has not been demonstrated. Antigens taken up by the mannose receptor are then delivered to endocytic compartments where they can be processed and eventually presented on CD1 (Prigozy *et al.*, 1997).

The mannose receptor is a membrane lectin or sugar specific receptor that is primarily expressed on macrophages and dendritic cells (Ezekowitz *et al.*, 1990). It is a highly efficient mechanism of antigen uptake, mediating the binding and endocytosis of mannose and fucose terminal glycoproteins (Avrameas *et al.*, 1996; Stahl and Ezekowitz, 1998). The mannose receptor is unusual in that it is recycled to the cell surface allowing for it to be used multiple times as compared to other receptors that are degraded along with their ligand (Tan *et al.*, 1997). Mannose receptors direct their antigen to endosomal compartments (MIIC) (Sallusto *et al.*, 1995) where they process antigen for eventual presentation to T cells (Prigozy *et al.*, 1997). This is a highly efficient process. Dendritic cells have been demonstrated to present mannosylated antigen 100-1000 fold more efficiently than non-mannosylated antigen (Engering *et al.*, 1997).

Mannose receptor contributes to uptake and presentation of glycosylated antigen particularly that originating from yeast and bacteria (Reis e Sousa *et al.*, 1993; Sallusto *et al.*, 1995). It has also been shown to mediate phagocytosis of various microorganisms that have exposed mannosylated glycoproteins. This receptor has been observed taking up

Candida albicans (Ezekowitz *et al.*, 1990), *Pneumocystis carini* (Ezekowitz *et al.*, 1991), *Mycobacterium sp.* (Schlesinger, 1993), and *Leishmania sp.* (Wilson and Pearson, 1988).

Because the cell wall of *C. neoformans* expresses a high level of carbohydrate in the form of mannan, glucan and N-acetyl glucosamine (Reis *et al.*, 1986; Vartivaran *et al.*, 1989), and because dendritic cells are known to express the mannose receptor it was investigated as a likely mechanism of interaction. This receptor also seemed a likely candidate as binding and ingestion of capsular *C. neoformans* has been observed with the mannose receptor on macrophages (Cross and Bancroft, 1995). Further, this interaction on a macrophage leads to the induction of proinflammatory cytokines including TNF- α , IL-1 β and MIP-1 α (Cross and Bancroft, 1995). This novel pathway of uptake and generating T cells responses is still not well defined and has never been described using freshly isolated dendritic cells or where MHC I and II presentation occurs.

Pirgozy and colleagues (1997) were the first to directly link recognition of a microbial antigen by the mannose receptor of the innate immune system to the induction of an adaptive T cell response. Here presentation was by CD1b. Studies here on *C. neoformans* reveal that the mannose receptor is important in the generation of T cell responses for this pathogen. This is the first report of a microbe using the mannose receptor on dendritic cells to elicit a T cell response.

The current studies demonstrate that DC are potent APC for *C. neoformans*. An abundance of evidence shows that dendritic cells can take up, and ultimately initiate T cell responses to *C. neoformans*. Dendritic cells are uniquely suited for the antigen presentation of fungal pathogens such as *C. neoformans* since they possess receptors that

make the uptake of yeast highly efficient. Further, these receptors can target antigen to MHC vesicles for processing and peptide binding to MHC, and recycle for continuous antigen uptake.

4.3.3 Organ Specific Responses

Cryptococcus enters the lung via inhalation. Therefore cells in the airway would represent the first site of contact with the cells of the immune system. Although dendritic cells are found to be the most important APC for *C. neoformans*, cells were acquired from bronchoalveolar lavage (BAL) to investigate whether cells obtained from the lung had any APC capacity. This cell population includes a large percentage of alveolar macrophages and a small proportion of dendritic cells (Van Haarst *et al.*, 1994). Alveolar macrophages would represent the first phagocytic cells that would be encountered by *C. neoformans*.

Alveolar macrophages

Alveolar macrophages are generally felt to be cells whose main function is to take up particulate matter (Green and Kass, 1963). They are not believed to be important in generating T cell responses both because of their suppressive nature (McCombs *et al.*, 1982; Toews *et al.*, 1984) and because they do not migrate to secondary lymphoid organs (Havenith *et al.*, 1993a, 1993b). There is some evidence that alveolar macrophages may be capable of presenting acapsular strains of *C. neoformans* to T cells (Vecchiarelli *et*

al., 1994ab), although in these studies the population referred to as alveolar macrophages was never characterized, and may have contained a proportion of dendritic cells.

It is plausible that alveolar macrophages could engulf, process and present antigen directly to the few T cells in the airspace. Alveolar macrophages may also play a role in processing and presentation by transferring peptides to dendritic cells for migration and eventual T cell stimulation (Havenith *et al.*, 1993a). Dendritic cells within BAL could also be processing and presenting the microorganism themselves.

In these studies cells obtained by BAL were able to induce T cell responses to *C. neoformans*; however, they were much less potent than blood derived loosely adherent cells. This low level of stimulation could be due to suppressive alveolar macrophages impeding on the dendritic cell induced response, or alveolar macrophages could be doing the presenting to T cells, but are less potent.

Dendritic cells in the lung

Dendritic cells are present in the airway epithelium and lung parenchyma. This potent APC population can be obtained by BAL (Havenith *et al.*, 1994; Van Haarst *et al.*, 1994). DC are also present in the lung and can be obtained in BAL, especially under inflammatory conditions (Havenith *et al.*, 1992). The presence of bacteria causes a rapid influx of dendritic cells to the lung (McWilliams *et al.*, 1994). There they can acquire antigen, such as *C. neoformans*, and migrate to regional compartments, such as peripheral lymph nodes, for T cell priming (Sertl *et al.*, 1986; Holt and Schon-Hegrad, 1987; Banachereau and Steinman, 1998). Dendritic cell migration to a secondary lymphoid

organ from an inflammatory site, such as the lung, can occur via the lymphatics or the peripheral blood prior to secondary lymphoid organs and presentation to T cells (Larsen *et al.*, 1990 and Austyn *et al.*, 1993). During this migration dendritic cells mature, and become more immunostimulatory (Cumberbatch and Kimber, 1992; Roake *et al.*, 1995). It is possible that cryptococcal infection of the lung may activate dendritic cells to express costimulatory molecules, as this has been described for monocytes (Vecchiarelli *et al.*, 1998a). This would enhance presentation of cryptococcal antigen to T cells.

Two possibilities exist with regards to antigen processing of *C. neoformans* in the lung. Alveolar macrophages may have the ability to process and present *C. neoformans*; however, this seems unlikely due to their immunosuppressive nature and inability to migrate to regional compartments. Therefore they are not likely to be relevant to the induction of protective cell mediated immune responses. It is much more likely that dendritic cells, now shown to be the most potent APC for *C. neoformans*, (resident or emigrants) are the active APC in the lung, as well as in the peripheral blood during an infection.

CHAPTER 5

ANTIGEN PROCESSING

5.1 BACKGROUND

Antigen processing is the process of taking up foreign proteins and converting molecules from a non MHC binding form to MHC binding form (usually a peptide). Presentation of protein antigens to T lymphocytes requires that the antigen be broken down to smaller peptides. The degree and type of processing that is required depends upon the APC and the nature of the antigen. Complex antigens or natural proteins require processing (Unanue *et al.*, 1984), although the degree of processing depends upon the individual protein/antigen. Peptides, on the other hand, may bind directly to the antigen binding groove of MHC. The complex nature of *C. neoformans* would indicate that processing would be required.

C. neoformans is a large encapsulated yeast that invades host cells. In order to process *C. neoformans* the host must phagocytose the organism, or disrupt the organism extracellularly and then take up the disrupted protein. Previous experiments demonstrated that *C. neoformans* is internalized, and therefore antigen processing is likely to be intracellular. Little is known about the processing requirements of this large pathogen, and indeed most fungi.

Antigen processing cells may require vigorous processing capabilities to deal with *C. neoformans*. It is possible that the large size of the pathogen may overwhelm the cellular machinery, and interfere with components of processing (Chapter One). It has also been suggested that the chemical characteristics of certain carbohydrates, like the cryptococcal cell wall, could also interfere with antigen processing (Gonzalez-Fernandez *et al.*, 1997). As well, the ability of the organism to resist intracellular killing (Herrmann *et al.*, 1994; Lee *et al.*, 1995) may influence processing. Although, there is the potential that cryptococcal proteins may gain access to the endogenous processing pathway, it is more likely that the initial, and probably essential processing of the organism is by the lysosomal-endosomal pathway.

The observation that a dendritic cell is the APC for *C. neoformans* (Chapter 4) was surprising, as dendritic cells are considered to have both limited endocytic capacity and limited degradative capacity. For instance dendritic cells have very few lysosomes (Chain and Kaye, 1986), which would suggest that these cells have reduced proteolytic potential. To reconcile this apparent limited endocytic activity with their ability to present *C. neoformans* the nature of the processing pathway required to generate lymphocyte proliferation in response to *C. neoformans* was examined.

In general, upon internalization, antigen progresses through the endocytic pathway. It is within the endocytic compartments (endosomes, MIIC, lysosomes) that processing occurs. Two models of the endocytic pathway exist, a maturation model and a transport model (reviewed by Stoorvogel, 1993; Gruenberg and Maxwell, 1995). The maturation model predicts that early endosomes mature into late endosomes, gradually

changing their composition and acidity. Here antigen enters endocytic vesicles that fuse to become early endosomes. These vesicles then move to later endocytic locations of the prelysosomes and finally the lysosome. As internalized antigen proceeds along the maturation pathway different epitopes would be generated while they encounter increasing denaturing and proteolytic conditions. The second endocytic pathway model predicts that early and late endosomes are preexisting compartments that communicate via vesicular traffic. Carrier vesicles would actively transport antigen from one vesicle to another. This fits well with speculation that early endosomes and MIIC are separate compartments rather than connected (Lanzavecchia, 1996), which would allow antigen to be targeted to one compartment or the other depending upon uptake mechanisms employed. Antigens would then progress along the endocytic pathway to later endocytic structures. Whether a transport or maturation model is true, these various endocytic compartments have differences in composition (Barbey *et al.*, 1995).

“Early” endosomes are the first vesicles that antigen is found in. They have slight acidity, and contain recycled MHC molecules (Gruenberg and Maxfield, 1995). These compartments segregate proteins into those that recycle to the plasma membrane and those that go on in the processing pathway. MIIC are acidic prelysosomal vesicles found in abundance in dendritic cells (Nijman *et al.*, 1995). They contain MHC II, invariant chain, cathepsin D, DM molecules, and the mannose 6-phosphate receptor (Guagliardi *et al.*, 1990; Kleijmeer *et al.*, 1995; Nijman *et al.*, 1995). These are molecules involved in presentation, chaperoning MHC, processing antigen, editing peptides and transferring cathepsins, respectively. Within MIIC (early lysosomes) peptides can bind nascent MHC

class II molecules (Harding, 1993). Generally, antigen taken up by macropinocytosis is targeted to MIIC, while endocytosed antigen enters the early endosome. MIIC occur late in the endocytic pathway as demonstrated by the lag in appearance of an exogenous tracer (Kleijmeer *et al.*, 1995). The final endosomal compartment is the lysosome. They are the dense late endocytic structures with high levels of lysosomal proteases, where MHC II is degraded (Harding, 1993). Phagolysosomal compartments involvement in processing has been suggested only for processing of antigen expressed within microbes that require extensive degradation (Diment and Stahl, 1985).

Dendritic cells, which are the APC for *C. neoformans*, act as the surveillance system of the immune system, capturing and processing antigen; then maturing into an immunostimulatory phenotype and homing to T cell areas of lymphoid organs. Dendritic cells can capture proteins and concentrate them in endocytic vesicles for eventual presentation to antigen specific T cells (Nijman *et al.*, 1995; Sallusto and Lanzavecchia, 1994), trafficking antigen to MIIC. Endocytic processing by dendritic cells is highly regulated compared to other APC. Dendritic cells are unique in their ability to downregulate processing. As the dendritic cell matures, they lose their endocytic and processing abilities, in part by the loss of acidic organelles (especially early endosomes) (Stossel *et al.*, 1990). Then during maturation of dendritic cells, MIIC convert to non-lysosomal vesicles that discharge MHC-peptide to the cell surface (Cella *et al.*, 1997). These large endocytic vesicles have a weak pH and are felt to be a site of antigen storage while the cell migrates from the site where the antigen is acquired to T cell areas of the afferent lymph nodes (Rescigno *et al.*, 1997). B cells and macrophages do not have this

ability to retain antigen (Davidson and Watts, 1989; Pure *et al.*, 1990; Kleijmeer *et al.*, 1995). This process may be very important as acquired peptides could be displaced by self peptides if antigen processing continued. Cryptococcal antigen acquired within the lung could then travel to sites of T cell stimulation before being completely degraded. In immature dendritic cells membrane MHC II is rapidly internalized, but the half life of these molecules increases dramatically once the cells mature (Cella *et al.*, 1997).

Dendritic cells and macrophages are unique in their use of the mannose receptor for antigen uptake. Mannose receptors and MHC II molecules co-localized to different compartments. The mannose receptor does not reach MIIC while mannosylated antigen does. It releases its ligand in an early endosomal structure. Mannosylated antigen then transits from this early compartment to a processing and loading compartment (Engering *et al.*, 1997; Tan *et al.*, 1997).

Early and late lysosomes are the organelles responsible for the major part of an antigen presenting cell's degradative function. A limited group of proteases are required for degradation of an antigen or for production of different antigenic determinants from an antigen (Demotz *et al.*, 1989; Demotz and Peleraux, 1996). These proteases are termed cathepsins, which are acid-optimal proteases that belong to two major families, the aspartic and cysteine proteases (Kirschke and Barrett, 1985). Cathepsin D and E are aspartic proteases, while B, L, H and S are cysteine proteases. All function optimally at an acidic pH, and are inactive at neutral pH (Fineschi and Miller, 1997). They also differ in their target amino acids (Chapman, 1998). Aspartic proteases are required in the early stages of antigen processing, while serine/cysteine proteases are required in later stages of

processing and invariant chain degradation (Maric *et al.*, 1994). Cysteine proteases act by catalysis which occurs through the formation of an intermediate complex between the enzyme and substrate (cysteine, histine and asparagine residues). The latter two residues polarize the cysteine to form the attacking nucleophile toward the carbonyl carbon in the targeted amide bond positions within the cleft. The resultant acyl enzyme complex is resolved by subsequent acid hydrolysis (Rawlings and Barratt, 1994). Cathepsin L targets leucine/aromatic amino acids at P1 and P1' (amino acids relative to the target bond). The enzyme cleavage pattern of cathepsin B is complex, with the best substrates containing basic amino acid residues. Aspartic proteases act in a similar fashion. The carboxyl group active site is not nucleophilic, instead a H-bonded water molecules attacks the carbonyl carbon (Fineschi and Miller, 1997). Cathepsin D and E cleave peptide bonds between hydrophobic amino acids (Fineschi and Miller, 1997). Cathepsin D specifically targets leucine aromatic amino acids at P2 and P1 (Chapman, 1998). The site for cathepsin E is unknown. Differences in active sites and differences in proteases available will have bearing on the ability of different APC populations to handle certain antigens as has previously been discussed (Vidard *et al.*, 1991; Zhong *et al.*, 1997; Chapter 1).

Very little is known about the processing capabilities of dendritic cells. The specific protease profile of dendritic cells has not been completely elucidated. Processing for presentation by MHC I and II antigens in dendritic cells has been inhibited by lysosomotropic agents, suggesting an acidic compartment is utilized (Svensson *et al.*, 1997). Presence of cathepsin D in dendritic cells is well documented (Kleijmeer *et al.*, 1995; Larsson *et al.*, 1997; Lutz *et al.*, 1997). Cathepsin D has been speculated to be

important in the processing of OVA, dextran and human serum albumin by murine dendritic cells (Rhodes and Anderson, 1993). Little is known, however, about the presence of other proteases in dendritic cells. Due to the limited information about dendritic cells and processing their ability to process and the proteases involved in processing a microbe like *C. neoformans* is difficult even to speculate.

Studies by Collins and Bancroft (1991), provide the only information available on the processing requirements of *C. neoformans*. In a murine system they found the organisms required processing for presentation to T cells. A lysosomal pathway of degradation was employed for the generation of immunogenic peptides, as disruption of lysosomes by chloroquine, inhibited the generation of lymphocyte proliferation. These studies, however, leave many questions about the processing of *C. neoformans* unanswered. First, the processing requirements of this microbe have never been examined in a human system. Further, the requirement of specific proteases have never been examined. Finally, most processing studies including the report by Collins and Bancroft (1991) have investigated either B cells and macrophages as the APC. Here (Chapter 4), a dendritic cell has been identified as the primary APC for *Cryptococcus*. Very little is known about antigen/microbe processing requirements in a system where dendritic cells are the APC.

Interfering with different steps in processing is a way to investigate processing requirements, and can be accomplished by inactivating APC, abolishing all proteolytic activity, or inhibiting individual classes of proteases. A variety of tools are able to accomplish this. APC that are fixed with paraformaldehyde are not capable of processing

protein antigens, but can present most mitogens and superantigens (Moreno and Lipsky, 1986). Fixed APC can also present peptides that bind directly to the presenting cell, since viable MHC mismatched APC release bacterial peptides for presentation by fixed MHC-matched APC to T cells (Pfeifer *et al.*, 1993). Specifically, fixation results in the cross linking of lysine residues by reaction with free amino groups (Pancake and Nathenson, 1973), rendering the cells metabolically inactive. Thus, paraformaldehyde fixation will block processing, but not presentation of antigens.

Protease inhibitors can affect processing in a number of ways. They can block the creation of epitopes, block presentation of peptides by interfering with binding to MHC, or enhance presentation of epitopes if they block an irrelevant or destructive pathway (Vidard *et al.*, 1991). Leupeptin blocks the activity of serine/cysteine proteases including cathepsins B, H, and L. Antipain is also a serine/cysteine protease inhibitor and inhibits the activity of cathepsins B and A. Pepstatin A is an aspartic protease inhibitor and blocks the activity of cathepsin D (Streicher *et al.*, 1984; Berzofsky *et al.*, 1988). Since the degree of processing depends on the individual antigen, selective protease inhibitors can be a valuable tool to separate the processing requirements.

Some antigens require only limited degradation, and therefore a limited panel of proteases (Legaard *et al.*, 1991; Bauer *et al.*, 1988). For instance, *Pseudomonas* exotoxin A-induced lymphoproliferation was inhibited by pepstatin A, an acid protease inhibitor while leupeptin a serine/cysteine inhibitor had no effect (Legaard *et al.*, 1992). Leupeptin has also been shown to inhibit I-E antigen restricted presentation of lysozyme, but not I-A restricted presentation (Puri *et al.*, 1986). Puri and Factorovich (1988) have demonstrated

that each antigenic fragment of the model antigen hen egg lysozyme (HEL) is produced by a unique set of proteases, antipain may block the generation of one peptide fragment and leupeptin another. Each antigen and MHC combination determines the proteases required to generate antigen or fragments. By utilizing a variety of protease inhibitors one can determine which proteases are active in the liberation of the stimulatory cryptococcal antigen.

T cell microfilaments also play an important role in the generation of antigenic peptides. Microfilaments are felt to play a role in antigen processing by their involvement in intracellular transport events rather than uptake and delivery. They are required for delivery of antigen to appropriate degradative sites and the site of peptide loading. Microfilament mediated vesicular transport of peptide antigen, MHC II and other cofactors is required for antigen processing by B lymphoblastoid cells (Soreng *et al.*, 1995). Cytochalasin B is an inhibitor of microfilament polymerization that inhibits intracellular transport, and can inhibit antigen processing (Geppert and Lipsky, 1990). Very few studies on the roles of microfilaments in processing by dendritic cells exist. Cytochalasin B inhibited receptor mediated endocytosis by dendritic cells, but had no effect on fluid phase and receptor mediated uptake by B cells (Lutz *et al.*, 1997; Rescigno *et al.*, 1997).

To determine the nature/requirements of antigen processing, APC were fixed to determine whether a metabolically active APC was required. To investigate the nature of the processing, lysosomotropic agents were employed to disrupt lysosomes. Specific elements of processing were studied by using protease inhibitors. The effect of

cytochalasin B was studied to understand the importance of microfilament polymerization in the production of lymphocyte proliferation.

5.2 RESULTS

5.2.1 Requirement for a metabolically active APC

C. neoformans likely behaves as a recall antigen that requires processing to produce T cell responses (Miller and Puck, 1984; Collins and Bancroft, 1991; Mody *et al.*, 1993). To determine if processing was required, the ability of fixed APC to present *C. neoformans* to T cells was examined. Loosely adherent cells (10^5) were lightly fixed in 1% paraformaldehyde prior to being placed in T cell cultures with *C. neoformans*. Lightly fixing antigen presenting cells completely abrogated the T cell proliferative response to *C. neoformans* (Figure 29A). These fixed cells, however, could function as APC when presentation but not processing was required, such as to the superantigen SEB (Legaard *et al.*, 1991) (Figure 29B). These data suggests that processing was required to release antigenic components of *C. neoformans*.

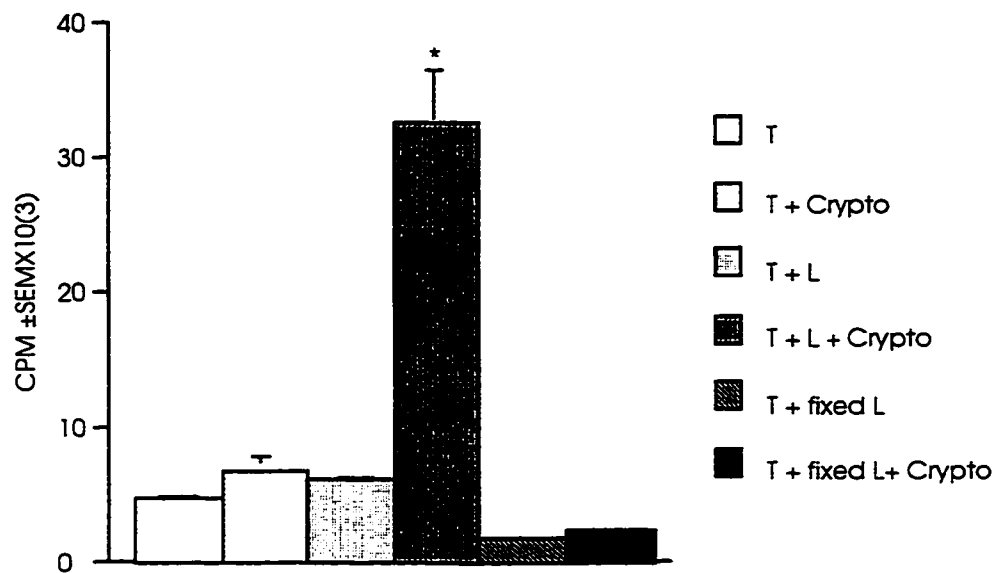
5.2.2 *C. neoformans* requires lysosomal processing

Having determined that processing was required to generate a proliferative response, the contribution of the lysosome was investigated to determine whether lysosomal processing was essential for the generation of T cell responses to *C. neoformans*. To do this a lysosomotropic agent was used. Lysosomotropic agents produce an alkaline environment in the lysosome, therefore processing of peptide antigens can not occur (Zeigler and Unaune, 1982). To assess the importance of lysosomal processing, PBMC were put in culture in the presence or absence of the

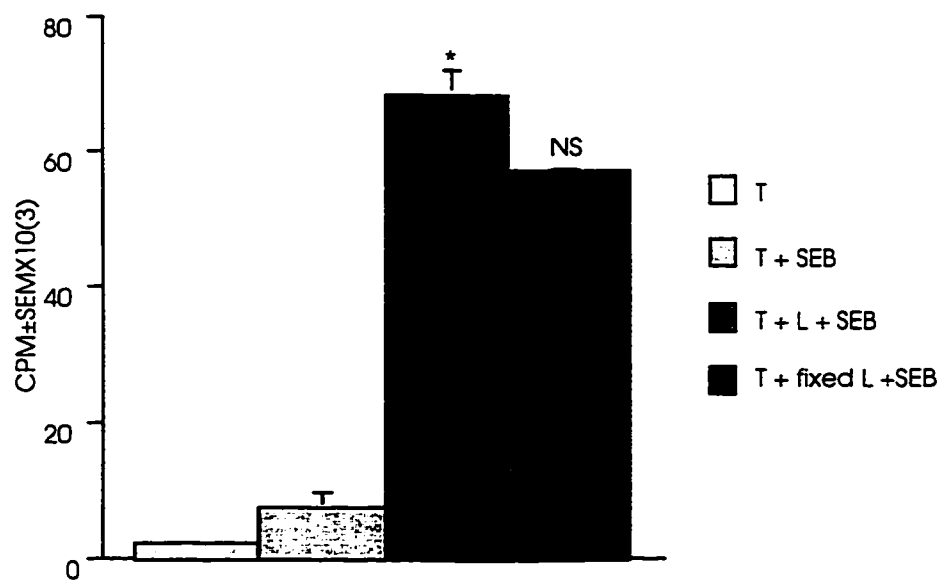
Figure 29A: Paraformaldehyde fixation of APC inhibits lymphocyte proliferation to *C. neoformans*. T cells (2×10^5 /well) were cultured in the presence of *C. neoformans* (2×10^5 /well) with loosely adherent cells (1×10^5 /well) that had been irradiated (L) or that had been lightly fixed (Fixed L, 1% paraformaldehyde for 5 minutes). Seven days later lymphocyte proliferation was assessed by thymidine incorporation. * $p < 0.05$ as compared to unstimulated control. One of 3 representative experiments.

Figure 29B: Paraformaldehyde fixed APC can elicit lymphocyte proliferation to a superantigen. T cells (2×10^5 /well) were cultured in the presence of SEB (0.5 $\mu\text{g/ml}$) with loosely adherent cells (1×10^5 /well) that had been irradiated (L) or that had been lightly fixed (Fixed L, 1% paraformaldehyde for 5 minutes). Thymidine incorporation was assessed 5 days later. NS- not significantly different as compared to stimulated cells. The experiment was repeated with similar results.

29A



29B



lysosomotropic agent ammonium chloride (NH_4Cl). NH_4Cl inhibits protein degradation without affecting antigen uptake or ingestion (Zeigler and Unanue, 1981). It can also inhibit phagosome-lysosome fusion (Gordon *et al.*, 1980). NH_4Cl inhibited lymphocyte proliferative responses to *C. neoformans* (Figure 30A). In control experiments, NH_4Cl had no effect on responses to the superantigen SEB (Figure 30B), which does not require lysosomal processing (Legaard *et al.*, 1991). These data suggests that lysosomal processing is required to liberate the antigenic components of *C. neoformans*.

5.2.3 Role of cathepsins B and D in processing of *C. neoformans*

Antigen processing has been shown to occur through the actions of endosomal and lysosomal enzymes, the cathepsins (Fineschi and Miller, 1997). Knowing lysosomal processing was required, the specific class of the protease was investigated to further analyze the fate of the antigen within the APC. Protease inhibitors can block processing of peptide antigens, thus, revealing the antigen specific nature of processing requirements (Takahashi *et al.*, 1989). The antigen itself plays a role in determining its susceptibility to different proteases (Jacquier-Sarlin *et al.*, 1995). Due to the complex nature of the cryptococcal cell wall, and the requirement to liberate proteins, and degrade them to peptides, *C. neoformans* will likely require multiple levels of processing.

Pepstatin A is an aspartic protease inhibitor (Buus and Werdelin, 1986). This inhibitor diminished lymphocyte proliferative responses to *C. neoformans*. At concentrations as low as 10 $\mu\text{g}/\text{ml}$, inhibition of lymphocyte proliferation was observed. Lymphocyte proliferation was completely abrogated at a concentration of 100 $\mu\text{g}/\text{ml}$

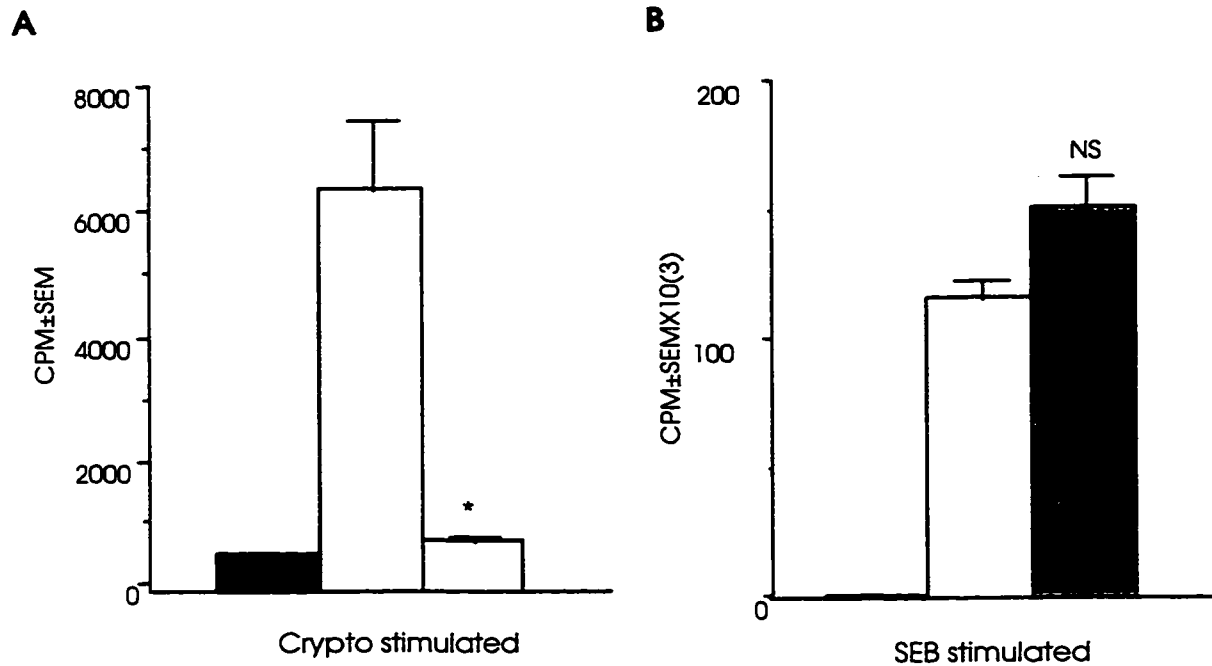


Figure 30: An NH₄Cl sensitive pathway is used to process *C. neoformans*. PBMC (2×10^5 /well) were stimulated with *C. neoformans* (2×10^5 /well) in the presence or absence of NH₄Cl (1 mM) for 7 days (Panel A); or PBMC were stimulated with SEB (1 μ g/ml) in the presence of NH₄Cl (1 mM) for 5 days (Panel B). ■ Stimulated PBMC, □ Stimulated PBMC + NH₄Cl. One of 4 representative experiments. * $p < 0.05$ as compared to stimulated cells. NS- as compared to stimulated cells.

(Figure 31A). In control experiments, this inhibitor had no effect on the response to the superantigen SEB (Figure 31B); demonstrating that the effects on *C. neoformans* were not due to nonspecific toxicity. These results suggest that type D cathepsin is involved in processing of *C. neoformans*.

Leupeptin and antipain are both serine/cysteine protease inhibitors (Streicher *et al.*, 1984). To examine the requirements for serine proteases on the liberation of the antigenic component of *C. neoformans*, these inhibitors were put into culture with PBMC and *C. neoformans*. Both leupeptin and antipain reduced lymphocyte proliferative responses (Figure 32A) showing that serine proteases were required to liberate the antigenic component of *C. neoformans*. These inhibitors showed an effect at as low a concentration as 1 $\mu\text{g}/\text{ml}$, and there was a dose dependent decrease in lymphocyte responses at 10-100 $\mu\text{g}/\text{ml}$. In control experiments, neither of these protease inhibitors (at 100 $\mu\text{g}/\text{ml}$) were able to inhibit the responses to a superantigen (SEB), which does not require processing for a T cell response (Figure 32B). Thus, processing of *C. neoformans* requires the participation of both serine and acid proteases for optimal breakdown of the organism for generation of proliferative responses. This would most likely include cathepsin B and D, which are inhibited by antipain/leupeptin and pepstatin A, respectively.

5.2.4 Role of microfilament polymerization in processing of *C. neoformans*

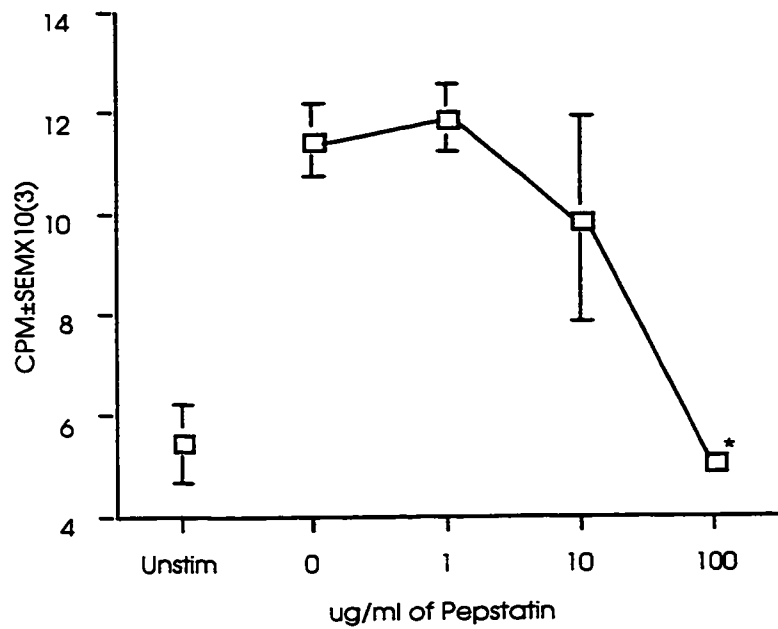
Microfilaments are disrupted by cytochalasins. Cytochalasin B effects microfilament dependent transfer of peptide antigen, which is a late step in antigen processing. Cytochalasin does not usually inhibit uptake or delivery of endocytosed

Figure 31A: Aspartic proteases are required for processing *C. neoformans*. PBMC (2×10^5 /well) were stimulated with *C. neoformans* (2×10^5 /well) in the presence or absence of various concentrations (0, 1, 10 or 100 $\mu\text{g/ml}$) of the protease inhibitor pepstatin A. Thymidine incorporation was determined 7 days later. One of three representative experiments. * $p < 0.05$ as compared to cells stimulated in the absence of inhibitor.

Figure 31B: Pepstatin A had no effect on lymphocyte proliferation to SEB. PBMC (2×10^5 /well) were stimulated with SEB (1 $\mu\text{g/ml}$) in the presence or absence of the protease inhibitor pepstatin A (100 $\mu\text{g/ml}$). Thymidine incorporation was determined 5 days later. One of three representative.

NS - not significantly different from PBMC stimulated in the absence of pepstatin A.

31A



31B

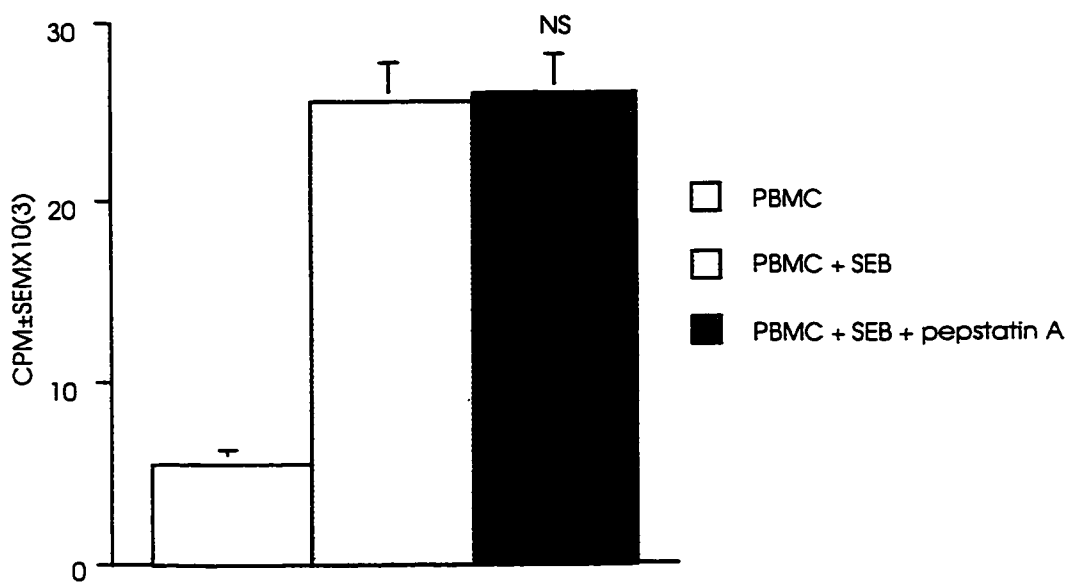
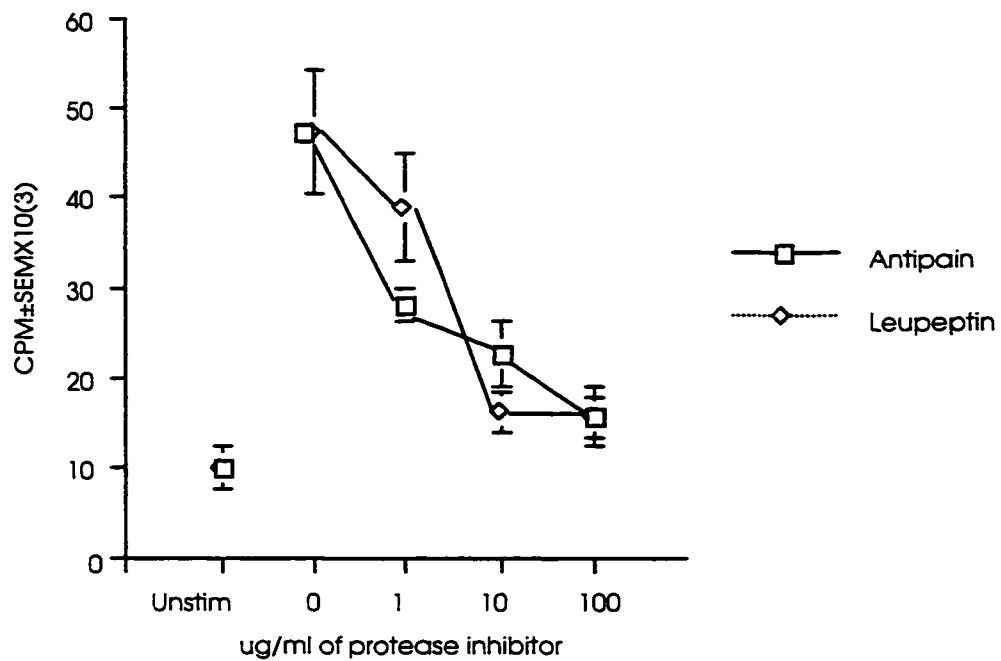


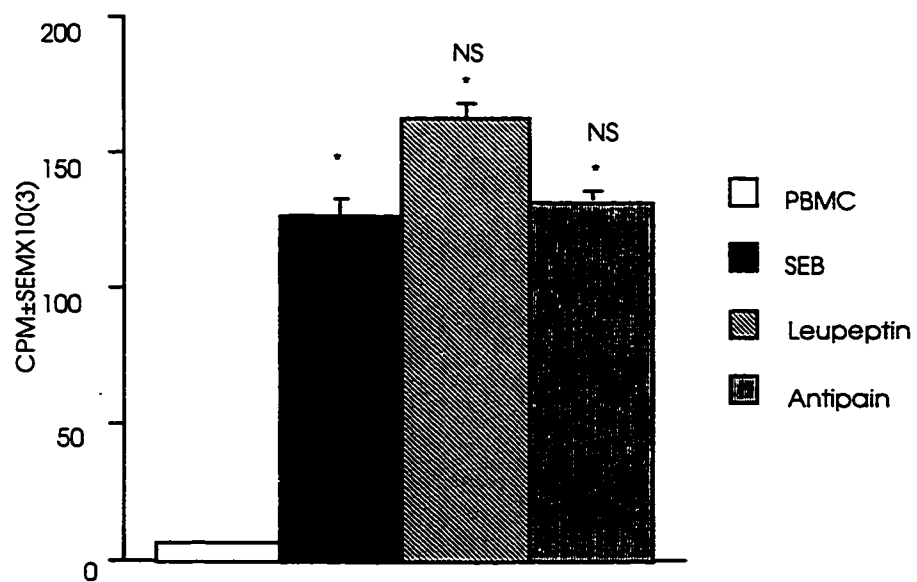
Figure 32A: Serine proteases are required for processing of *C. neoformans*. PBMC (2×10^5 /well) were stimulated with *C. neoformans* (2×10^5 /well) in the presence or absence of various concentrations (0, 1, 10 or 100 $\mu\text{g}/\text{ml}$) of the protease inhibitors leupeptin or antipain. Thymidine incorporation was determined 7 days later. * $p < 0.05$ as compared to cells stimulated in the absence of inhibitors. One of four representative experiments

Figure 32B: Serine protease inhibitors have no effect on lymphocyte proliferation to SEB. PBMC (2×10^5 /well) were stimulated with SEB (1 $\mu\text{g}/\text{ml}$) in the presence or absence of (100 $\mu\text{g}/\text{ml}$) of the protease inhibitors leupeptin or antipain. Thymidine incorporation was determined 5 days later. * $p < 0.05$ as compared to unstimulated cells * $p < 0.05$ as compared to unstimulated control. NS - not significant as compared to PBMC stimulated with SEB. One of four representative experiments.

32A



32B



protein to lysosomes, which is an early stage of antigen processing (Soreng *et al.*, 1995).

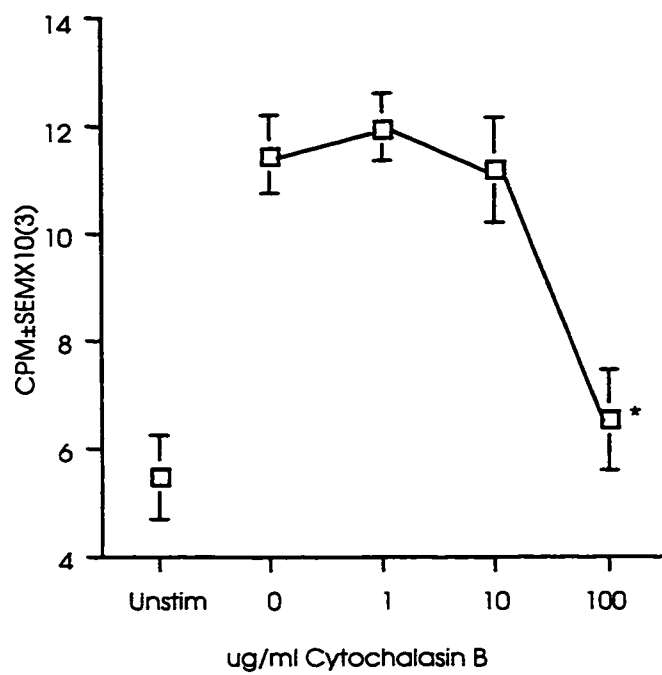
To investigate whether microfilament polymerization was required for processing, and ultimately the generation of T cell responses to *C. neoformans*, varying concentrations of cytochalasin B were added to culture. Cytochalasin B was found to diminish proliferative responses to *C. neoformans*, at concentrations greater than 10 $\mu\text{g/ml}$ (Figure 33A). In control experiments a high concentration cytochalasin B abrogated responses to the superantigen SEB as expected (Figure 33B; Geppert and Lipsky, 1990). Thus, microfilament polymerization is required for the generation of productive proliferative responses to whole *C. neoformans*.

Figure 33A: Microfilament polymerization is required for lymphocyte proliferation to *C. neoformans*. PBMC (2×10^5 /well) were stimulated with *C. neoformans* (2×10^5 /well) in the presence or absence of various concentrations (0, 1, 10 or 100 $\mu\text{g}/\text{ml}$) of cytochalasin B. Thymidine incorporation was determined 7 days later. * $p < 0.05$ as compared to cells stimulated in the absence of cytochalasin B. One of three representative experiments.

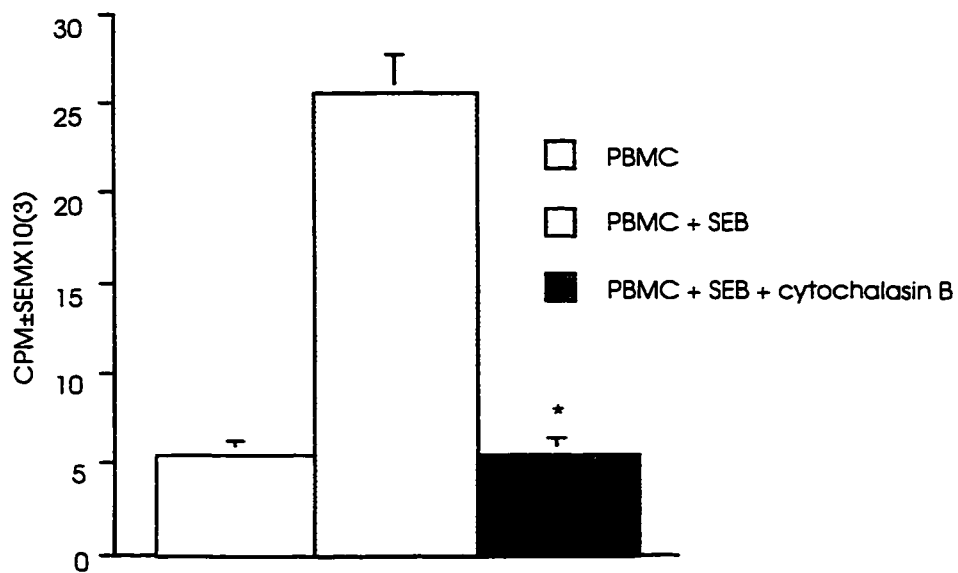
Figure 33B: Cytochalasin B abrogated lymphocyte proliferation in response to SEB. PBMC (2×10^5 /well) were stimulated with SEB (1 $\mu\text{g}/\text{ml}$) in the presence or absence of cytochalasin B (100 $\mu\text{g}/\text{ml}$). Thymidine incorporation was determined 5 days later. One of three representative.

* $p < 0.05$ as compared to cells stimulated in the absence of cytochalasin B.

33A



33B



5.3 DISCUSSION

A number of observations were made about antigen processing requirements of *C. neoformans* 1) a metabolically active APC was required 2) lysosomal processing was required 3) aspartic proteases, serine/cysteine proteases were involved in the generation of antigenic fragments 4) microfilament polymerization contributed to the formation of antigenic fragments.

Initially, experiments were carried out to assess whether any processing was required. There is some suggestion that *C. neoformans* behaves like a mitogen as proposed by Graybill and Alford (1974). Mitogens and superantigens generally do not require processing, while protein recall antigens do (Fleischer *et al.*, 1988). As fixed cells are not capable of processing protein antigen, experiments showed that *C. neoformans* requires a metabolically active APC capable of processing antigen. The current experiments also demonstrate that *C. neoformans* does not require limited processing like the superantigens *Mycoplasma arthritidis* supernatant or *Pseudomonas* exotoxin A (Bauer *et al.*, 1982; Legaard *et al.*, 1991). These data is most consistent with *C. neoformans* stimulating T cells as an antigen.

Lysosomotropic agents such as ammonium chloride increase the pH of acid intracellular vesicles and decrease the degradation of endocytosed proteins (Zeigler and Unanue, 1982). NH_4Cl has previously been shown not to effect the uptake of microbes (*Listeria monocytogenes*) (Zeigler and Unanue, 1982), or effect transport of MHC class II expression on the cell surface (Soreng et al ,1995). Most lysosomal proteases require an

acidic environment to function optimally. If NH_4Cl has an effect, then proteins have to be internalized into acid bearing vesicles to be processed. The current experiments suggest that degradation within acidic intracellular vesicles is a prerequisite for successful lymphocyte proliferation to occur in response to *C. neoformans*. Thus, uptake into early acidic vesicles by dendritic cells would be essential. Only one other study has investigated the effects of lysosomotropic agents where dendritic cells were the APC. Here NH_4Cl treatment of langerhans cells abrogated protein processing (Stossel *et al.*, 1990).

Dendritic cells, however, do not have an abundance of proteolytic activity, and have a paucity of lysosomes (Steinman, 1991). The endocytic activity and processing ability for these cells is also small when compared to the size and activity of the vacuolar system of the macrophages, which extensively scavenge and degrade antigen (Steinman, 1991). It may be that dendritic cells use their limited proteolytic machinery more efficiently, or that they only require limited amounts of antigen and peptide to generate T cell responses as suggested by Swanson and Steinman (1996).

Because different APC populations have different protease compositions processing may differ with cell type (McCoy *et al.*, 1993) as previously discussed. Major differences in cellular content of cathepsins have been observed for different APC. For instance B cells do not express cathepsin L, while dendritic cells do (Lutz *et al.*, 1997).

Proteases belonging to different classes may participate in the processing of distinct antigenic peptides as different epitopes are generated depending on the inhibitors used (Puri and Factorovich, 1988). Leupeptin, antipain and pepstatin all inhibit

proliferative responses to *C. neoformans* supporting the requirement for B, and D cathepsins. Cathepsin B and D have both been co-localized with class II molecules in antigen processing compartments of dendritic cells (Kleijmeer *et al.*, 1995). When macrophages utilize this type of uptake the vesicle quickly binds with lysosomes and their abundant lysosomal enzymes (Racoosin and Swanson, 1993). The lysosome is an unlikely candidate for the site of antigen processing since the majority of material that reaches this compartment is degraded to a size too small to retain any antigenicity (Chain *et al.*, 1988). As the mannose receptor is utilized by *C. neoformans*, and the mannose receptor transports antigen to MIIC (Engering *et al.*, 1997; Pirgozy *et al.*, 1997; Tan *et al.*, 1997) then only resident D and B cathepsins would likely to be involved in processing (Guagliardi *et al.*, 1990).

Receptor binding can also effect antigen susceptibility to degradation by certain cathepsins. For example, if C3b is bound to tetanus toxoid degradation of the CR and tetanus is no longer susceptible to cathepsin B, but it is to cathepsin D (Jacquier-Sarlin *et al.*, 1995). The apparent utilization of the mannose receptor by *C. neoformans* suggests that it is unlikely that the receptor would protect epitopes or change processing, as the mannose receptor is recycled to the cell surface once in the early endosome (Engering *et al.*, 1997; Tan *et al.*, 1997). Thus, *C. neoformans* is likely processed in MIIC, as these compartments are late in the endocytic pathway and contain proteases (cathepsin B and D) whose activity when blocked inhibit the induction of lymphocyte proliferation.

Due to the numerous contributors to successful processing of *C. neoformans* it seems likely that immunogenic epitopes are “buried” and require harsh degradative

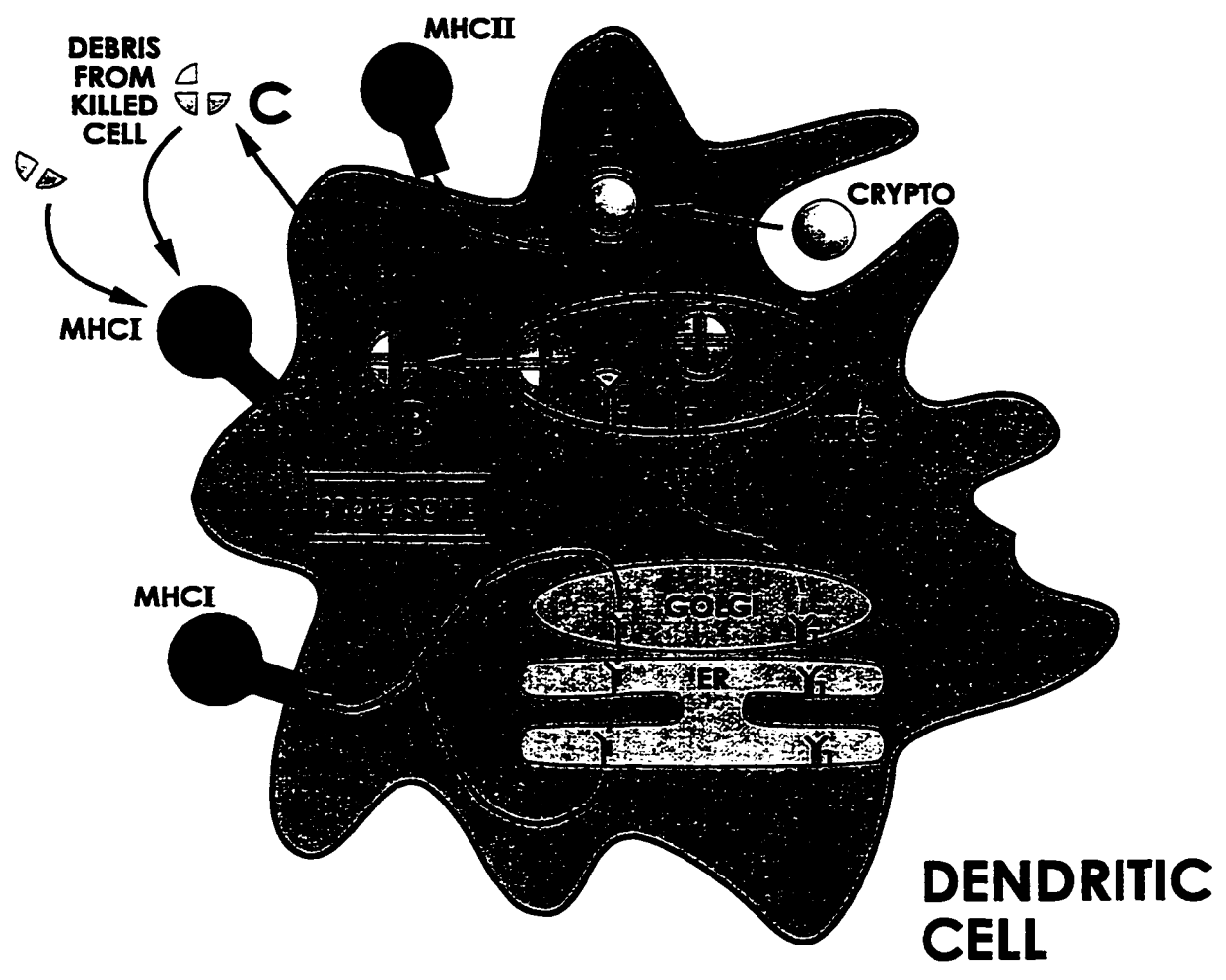
conditions (acidity and abundant proteases) of later endosomal compartments to be released. The use of the mannose receptor suggests peptides are ultimately presented on newly synthesized class II. If epitopes were more accessible they would likely require more limited proteolysis, and would be accessible earlier along the proteolytic pathway perhaps to recycled class II MHC from the plasma membrane (Demotz *et al.*, 1989).

Cytochalasin B depolymerizes actin filaments and can inhibit antigen processing (Soreng *et al.*, 1995). It does not inhibit antigen uptake or delivery of endocytosed proteins to lysosomes, but can effect catabolism of proteins, by effecting antigen transport for MHC binding (Soreng *et al.*, 1995). Cytochalasin B, however, has not been reported to inhibit receptor mediated endocytosis for B cells (Lutz *et al.*, 1997; Rescigno *et al.*, 1997). It was found to inhibit processing of *C. neoformans*. It is unclear, however, if the effect observed here is on antigen transport or antigen uptake.

The importance of lysosomal processing and lysosomal proteases for *C. neoformans* suggests that the processing required for generation of CD4 and CD8 T cell responses may overlap. Within a cell, processing follows two generalized pathways that have been previously reviewed (Chapter 1). These pathways are the class II dependent lysosomal pathway and the class I dependent pathway of processing by the proteasome. Degradation in the lysosome generates peptides that are eventually presented in context of MHC class II molecules to CD4 cells. Peptides generated by degradation within the proteasome bind to MHC class I molecules for presentation to CD8 cells. Processing of these peptides do not usually require lysosomal proteases (Morrison *et al.*, 1986). The current studies have investigated only the lysosomal pathway. Both CD4 and CD8

responses have been shown to be important in host defense to *C. neoformans* (Chapter 3). Dendritic cells are very efficient at stimulating T cell responses to exogenous and endogenous antigen (Steinman, 1991). CD4 and CD8 cell responses to *C. neoformans* could be generated by a number of processing pathways (Figure 34). Lysosomal processing has been shown to be important in generating responses to *C. neoformans*. The requirement for lysosomal processing fits well with the stereotypical (exogenous pathway) production of CD4 T cell responses (A). Since inhibiting lysosomal processing abrogates lymphocyte proliferation, it suggests that CD8 cell activating peptides may require some processing within a lysosomal compartment before they egress in the cytosol for subsequent further processing and ultimately class I presentation (B). For instance, fully mature dendritic cell macropinosomes, which are felt to store antigen, may release antigen into the cytosol to serve MHC I presentation (Nobury *et al.*, 1995). The whole organism may also gain access for the cytosol for class I presentation (B). This is known to occur for bacteria such as *Listeria monocytogenes* that can escape into the cytosol (Portnoy *et al.*, 1988), it has also been demonstrated for bacteria with no known mechanism for cytosolic penetration (*E. coli*; *S. typhimurium*) (Pfeifer *et al.*, 1993). It is speculated that organisms that can escape into the cytosol and those that are retained in a vacuolar compartment will use distinct mechanism of class I processing and presentation (Pfeifer *et al.*, 1993), but the exact mechanics of this are unknown. It is also plausible that regurgitation of processed peptides or the debris from killed organisms could be acquired and loaded on MHC I at the cell surface (Pfeifer *et al.*, 1993) (C). Since CD8

Figure 34: Possible processing pathways of *C. neoformans* for the generation of CD8 T cell responses



responses are dependent upon CD4 cells it is also possible that blocking presentation to CD4 cells would abrogate CD8 responses. Thus two possibilities exist, the pathogen may survive intracellularly and release secreted antigen into the processing pathway, or peptides processed by the endocytic pathway would get access to post golgi MHC I molecules either on other cells via peptide regurgitation or within vacuolar compartment using previously unoccupied MHC I.

Dendritic cells are known to be unique in their processing capabilities. They are able to down regulate processing and mature into an immunostimulatory phenotype. They are also unique in their time course of processing and ability to store antigen as they migrate to present antigen to T cells. Here it has been shown that in a system where dendritic cells are the APC, lysosomal processing is crucial, serine and aspartic proteases contribute to lymphocyte responses, and aggressive processing of this fungi is required. Dendritic cells have now been demonstrated to process large microbes using a lysosomal pathway. For the first time a role for aspartic and cysteine proteases has been demonstrated for dendritic cells.

In summary, these data here is supportive of the antigenic nature of *C. neoformans*. It demonstrates a requirement for aggressive processing to induce T cell responses. Knowledge of the processing requirements of *C. neoformans* may be important in helping to identify what are the important components for production of protective immune responses.

CHAPTER 6

POLYSACCHARIDE CAPSULE

6.1 BACKGROUND

Although human lymphocytes are stimulated by whole cryptococcal organisms (Graybill and Alford, 1974; Miller and Puck, 1984; Mody and Syme, 1993), the characteristics of *C. neoformans* that influence the lymphocyte response are largely unknown. The possibility was considered that these characteristics are the known virulence factors for *C. neoformans*.

Virulence of *C. neoformans* is partially determined by serotype. Infections due to serotype A organisms of *C. neoformans* var. *neoformans* are most common (Bennett *et al.*, 1977), implying that serotype A organisms have a selective advantage. It has been established that human lymphocytes proliferate in response to serotype A organisms (Miller and Puck, 1984), but not if this is unique to this serotype. Further, it is not known if serotype D organisms, which are a less common cause of infection in humans, produce a greater lymphocyte response than to organisms of serotype A. Studies were performed to examine the effect of serotype on lymphocyte proliferation.

The size of the cryptococcal polysaccharide capsule is another important virulence factor of *C. neoformans* (Mitchell and Friedman, 1972; Dykstra *et al.*, 1977;

Fromtling *et al.*, 1982). Murine studies demonstrate that cryptococcal polysaccharide (CPS) suppresses lymphocyte proliferation (Collins and Bancroft, 1991), and antibody formation (Murphy and Cozad, 1972; Breen *et al.*, 1982). As clonal proliferation of T cells is a hallmark of the cell mediated immune response, the possibility that CPS-mediated inhibition of lymphocyte proliferation may be an important virulence factor was considered.

Initial experiments examined the effect of CPS on lymphocyte proliferation as a measure of cell mediated immunity, by comparing responses of variously encapsulated strains and performing experiments in the presence or absence of exogenous polysaccharide. Once it was established that polysaccharide interfered with cell mediated responses the mechanism of this inhibition was investigated.

There are a number of mechanisms by which CPS may work to suppress the development of the lymphocyte response. CPS can induce immunosuppressive cytokines such as IL-10 (Vecchiarelli *et al.*, 1996), which suppresses antigen specific T cell proliferation, including responses to *C. neoformans*, by a number of mechanisms (de Waal Malefyt *et al.*, 1991; Del Prete *et al.*, 1993; Monari *et al.*, 1997). CPS can also cause shedding of cell surface receptors which are important in cell-cell interactions (Dong and Murphy, 1996), and could be involved in antigen presentation. CPS can also inhibit cryptococcal uptake by phagocytic cells (Mitchell and Friedman, 1972; Kozel *et al.*, 1988a). Thus, there is the potential for CPS to suppress the development of T cell responses by stimulating production of immunosuppressive cytokines, by inhibiting cell-

cell interactions necessary for antigen presentation to T cells, or by inhibiting the uptake of *C. neoformans* by the antigen presenting cell.

To determine whether CPS suppresses lymphocyte proliferation by production of IL-10, lymphocytes were stimulated with CPS-treated *C. neoformans* in the presence or absence of neutralizing antibody to IL-10. To determine if CPS was affecting antigen presenting cell/T cell interactions, CPS was added to the PBMC and excess was removed before stimulation with *C. neoformans*. To determine whether the antiphagocytic properties of polysaccharide contributed to a reduction in lymphocyte proliferation, phagocytosis was correlated with ³H-TdR incorporation.

Once it was established that diminished lymphocyte proliferation in the presence of CPS was due to a decrease in uptake, experiments were designed to assess whether antibody or human serum could augment the proliferative response. A variety of opsonic fragments are present in human serum, which could aid in uptake (Kozel and McGaw, 1979; Levitz and Dibenedetto, 1988; Levitz *et al.*, 1993). In a murine model, specific anti-cryptococcal antibodies can opsonize *C. neoformans* and augment uptake (Dromer *et al.*, 1989; Mukherjee *et al.*, 1995) and antibodies to glucoronxylmannan conjugated to tetanus toxoid promote phagocytosis of *C. neoformans* in the absence of complement (Zhong and Pirofski, 1996). Although antibody to capsular polysaccharide (CPS) can be protective (Mukherjee *et al.*, 1994; Pirofski *et al.*, 1995), there is little evidence that humoral mechanisms are important in natural host defense to *C. neoformans*. There are naturally occurring cryptococcal antibodies (Dromer *et al.*, 1988; Houtp *et al.*, 1994;

Deshaw and Pirofski, 1995), but these fail to opsonize *C. neoformans*. It seems reasonable then that if capsular polysaccharide impairs T cell responses (which are critical for anticryptococcal host defense), then specific anti-capsular antibodies might be able to enhance host defense to *C. neoformans* by enhancing uptake and ultimately presentation to T cells. Experiments were therefore performed to test the ability of complement or anticapsular antibody to ameliorate the effect of CPS on lymphocyte proliferation. Thus, two characteristics of *C. neoformans* that alter the human immune response were examined, by studying the influence of cryptococcal capsular polysaccharide, and serotype of *C. neoformans* on proliferation of peripheral blood mononuclear cells (PBMC) *in vitro*.

6.2 RESULTS

6.2.1 Serotype

Cryptococcal infections due to serotype A organism are the most common (Bennett *et al.*, 1977), which suggests they may have a selective advantage over serotype D organisms. It is possible that serotype D organisms generate a greater cell mediated immune response than serotype A organisms. Experiments were performed to determine if serotype D organisms stimulate the response to a greater degree than serotype A organisms. PBMC were stimulated by *C. neoformans* strain 613 (serotype D) and strain 68 (serotype A) which have similar capsule sizes. Lymphocytes proliferated in response to both strains. The stimulation index for serotype D *C. neoformans* was 3.36 ± 0.41 ; the stimulation index for serotype A *C. neoformans* was 3.94 ± 1.04 (n=4 subjects). The mean counts per minute \pm SEM (10^3) were 14.02 ± 4.56 and 14.13 ± 2.91 , respectively (Figure 35A). When two other strains (C3D and 3501) were compared it was also found that lymphocytes again responded to strains of both serotypes, and that there was no difference in the response (Figure 35B). Serotype D organisms did not stimulate the response to a greater degree than serotype A organisms. This suggests that serotype does not affect a measure of cell mediated immunity.

6.2.2 Polysaccharide Capsule

The capsular polysaccharide of *C. neoformans* is an important virulence factor. To determine the effect of the size the polysaccharide capsule on lymphocyte

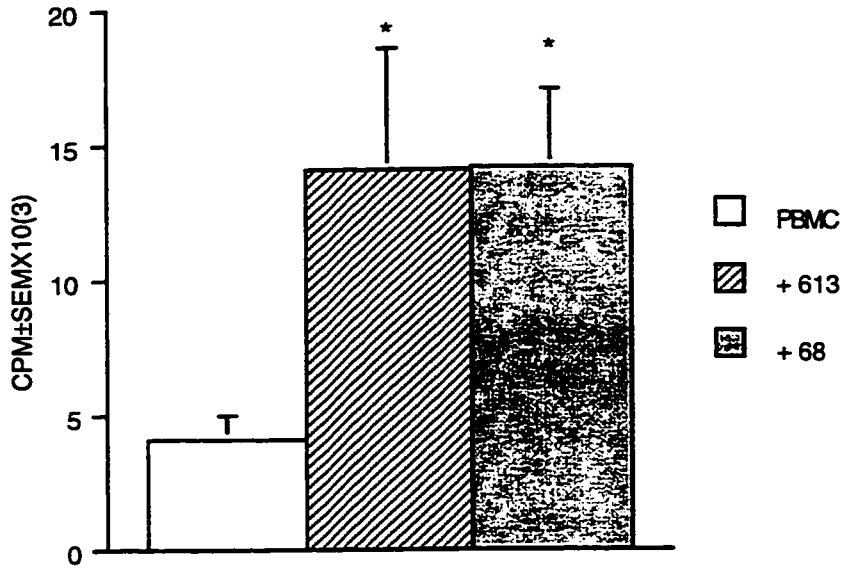
Figure 35: *C. neoformans* of serotype A and D can induce lymphocyte proliferation
PBMC (2×10^5 /well) were put into culture in the presence or absence of *C. neoformans*
(2×10^5 /well). Seven days later thymidine incorporation was assessed.

Panel A: Strains 613 (serotype D) and Strain 68 (serotype A).

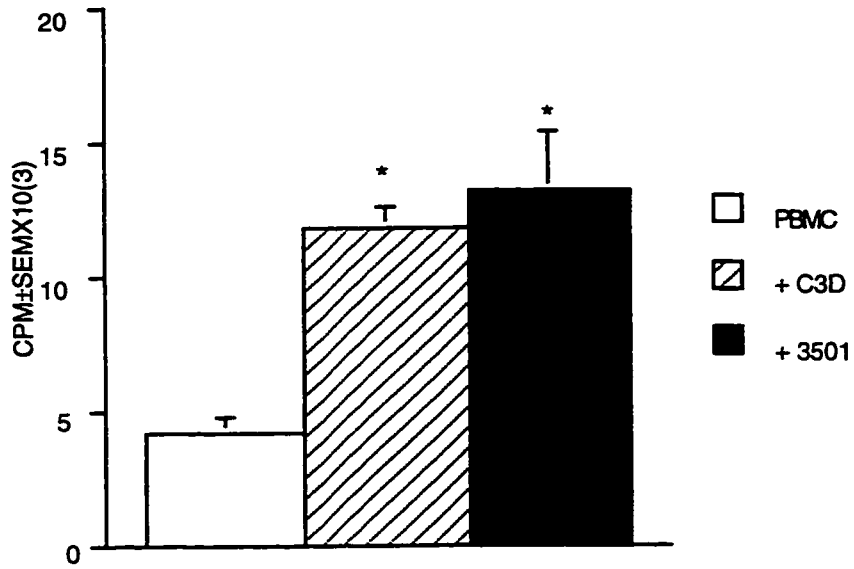
Panel B: Strains C3D (serotype A) and strain 3501 (serotype D).

Each graph represents the mean of four experiments. * $p < 0.05$ as compared to
unstimulated cells.

35A



35B



proliferation strains of *C. neoformans* that varied in capsule size were used to stimulate PBMC. Four strains of *C. neoformans* were used, all serotype A: strain C3D, which is minimally encapsulated; strain 68, which is lightly encapsulated; strain 145, which is moderately encapsulated; and strain 6, which is heavily encapsulated. All strains caused lymphocyte proliferation (Figure 36). The response was lowest for the most heavily encapsulated strain, and greatest for the minimally encapsulated strain. The magnitude of the proliferative response increased as the capsule size decreased.

Upon entry into the lung, acapsular *C. neoformans* begins to synthesize polysaccharide capsule (Fahri *et al.*, 1970). To understand whether the polysaccharide capsule of one organism could suppress the response to another organism the proliferative response to an acapsular strain was studied in the presence or absence of a heavily encapsulated strain. PBMC were stimulated with strain 67 (an acapsular mutant) in the presence or absence of strain 6 (a heavily encapsulated strain). Lymphocytes proliferated in response to strain 67, but minimally to strain 6. The addition of strain 6 to 67 had no effect on the proliferative response to strain 67 (Figure 37). Experiments were performed at two concentrations of the organism (1×10^5 /well and 2×10^5 /well) to ensure that the effect could not be attributed to the number of organisms in a well. Thus, heavily encapsulated strains can not effect the response to cryptococcal organisms that do not possess a polysaccharide capsule.

To determine whether polysaccharide capsule was responsible for suppressing the proliferative response, the proliferation of PBMC to an acapsular strain of *C. neoformans*

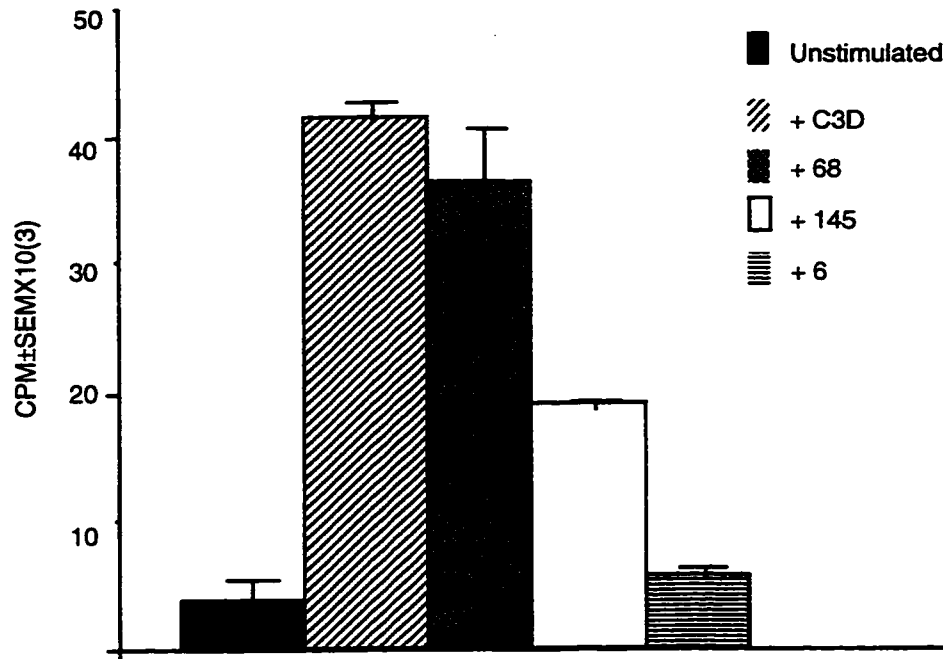


Figure 36: Effect of capsule size of *C. neoformans* on lymphocyte proliferation.

PBMC (2×10^5 /well) were cultured with 4 strains of *C. neoformans* (2×10^5 /well); strain C3D, minimally encapsulated; strain 68, lightly encapsulated; strain 145, moderately encapsulated; and strain 6, heavily encapsulated. Seven days later lymphocyte proliferation was assessed by thymidine incorporation. All strains were significantly different (ANOVA for four experiments, $p < 0.05$).

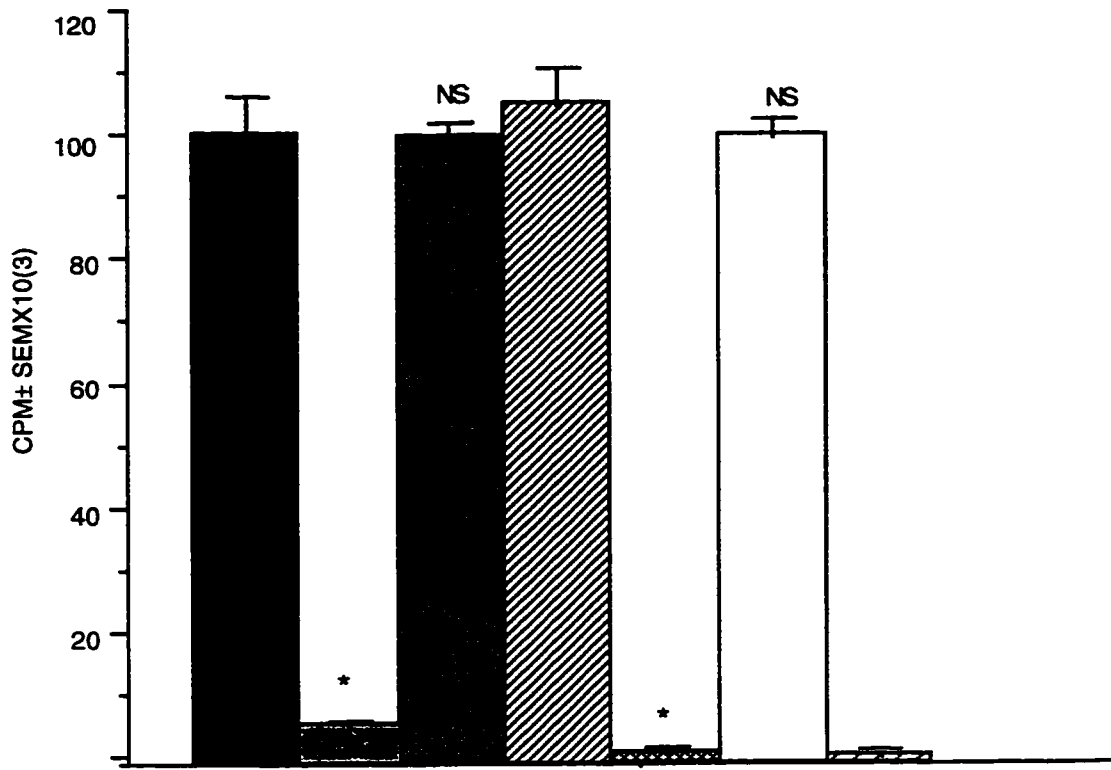


Figure 37: A heavily encapsulated strain of *C. neoformans* has no effect on the proliferative response of PBMC to an acapsular strain. PBMC were cultured with *C. neoformans* strain 67 alone (an acapsular mutant) at 1×10^5 /well (■); strain 6 alone (heavily encapsulated) at 1×10^5 /well (■); strain 67 at 1×10^5 /well with strain 6 at 1×10^5 /well (■); strain 67 at 2×10^5 /well, (▨); strain 6 at 2×10^5 /well (▩); strain 67 at 2×10^5 /well with strain 6 at 2×10^5 /well (□); or PBMC alone (▨). Lymphocyte proliferation was assessed seven days later by thymidine incorporation.

* $p < 0.001$ compared to strain 67 alone NS - not significantly different compared to strain 67 alone. One of two experiments.

(strain 67) was determined in the presence or absence of purified polysaccharide from serotype A and serotype D organisms. Lymphocytes proliferated briskly in response to strain 67; however, the response was suppressed after the addition of polysaccharide from either serotype at 250 $\mu\text{g}/\text{ml}$ (Figure 38 A and B). The response could be restored by reducing the amount of polysaccharide to 0.5 to 0.001 $\mu\text{g}/\text{ml}$. Thus, addition of exogenous polysaccharide could inhibit the response to an acapsular strain.

6.2.3 Mechanism of CPS induced Suppression

Having determined that CPS could influence lymphocyte proliferation it was important to determine the mechanism of this inhibition. There are a number of mechanisms by which the polysaccharide capsule may work to suppress the immune response. Cryptococcal polysaccharide has been shown to stimulate production of immunosuppressive cytokines such as IL-10 (Vecchiarelli *et al.*, 1996). Experiments were undertaken to determine if anti-inflammatory cytokines, produced as a result of polysaccharide capsule, were effecting lymphocyte proliferation. PBMC were put into culture in the presence or absence of acapsular *C. neoformans*, purified polysaccharide (50 $\mu\text{g}/\text{ml}$) and anti-IL-10 (10 $\mu\text{g}/\text{ml}$, ATCC). The presence of anti-IL-10 monoclonal antibody did not restore the lymphocyte proliferative response (Figure 39) suggesting that IL-10 is not responsible for the CPS induced suppression of lymphocyte proliferation. Polysaccharide may cause shedding of surface molecules on host cells (Dong and Murphy, 1996). Since surface molecules are important in the interactions

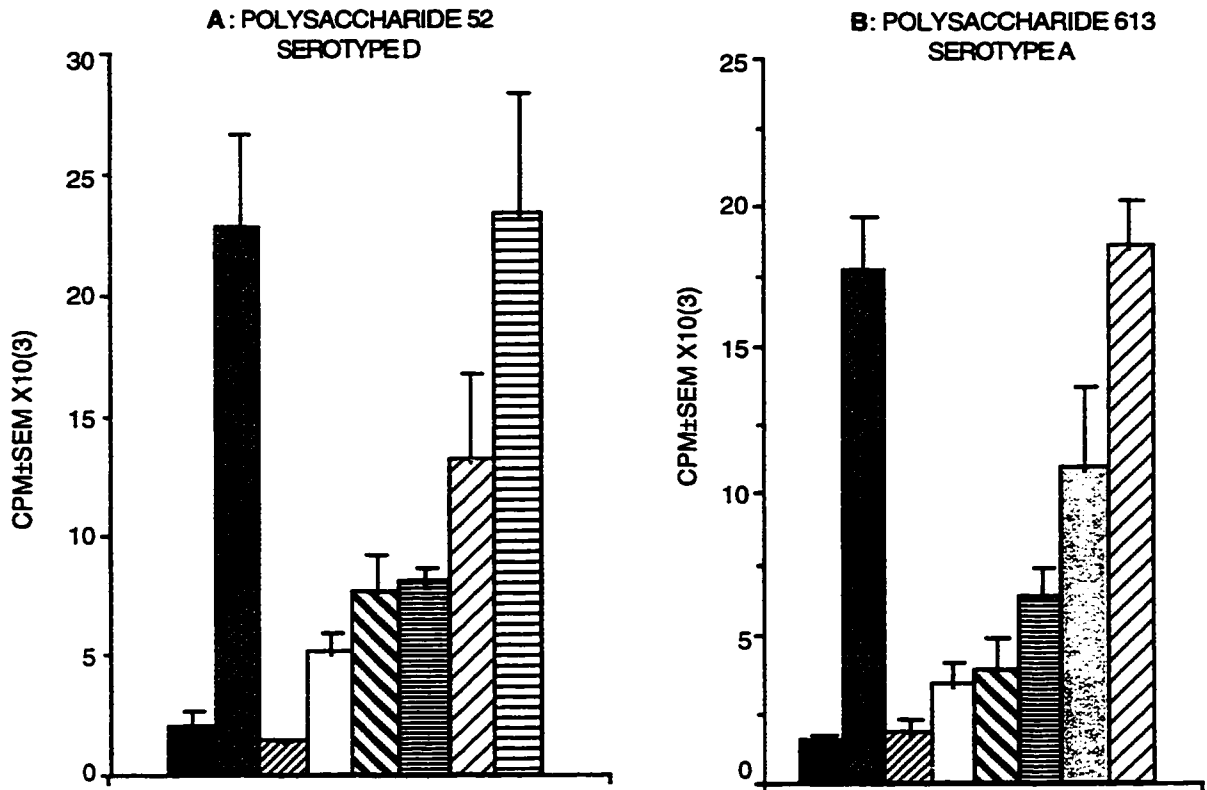


Figure 38: Purified polysaccharide capsule inhibits lymphocyte proliferation in response to acapsular *C. neoformans*: Panel A: polysaccharide capsule from strain 52 serotype D; Panel B: polysaccharide capsule from strain 613 serotype A. PBMC were cultured alone (■), with heat-killed *C. neoformans* strain Cap 67 (■), or with Cap 67 and polysaccharide capsule: 250 µg/ml (▨), 50 µg/ml (□), 25 µg/ml (▩), 5 µg/ml (▧), 2.5 µg/ml (▦), 0.5 µg/ml (▤), 0.005 µg/ml (▣). The experiment was repeated with equivalent results. There was a statistically significant increase in cpm in cultures containing decreasing amounts of polysaccharide ($r=0.831$, $p<0.001$ (Panel A); $r=0.816$, $p,0.001$ (Panel B)).

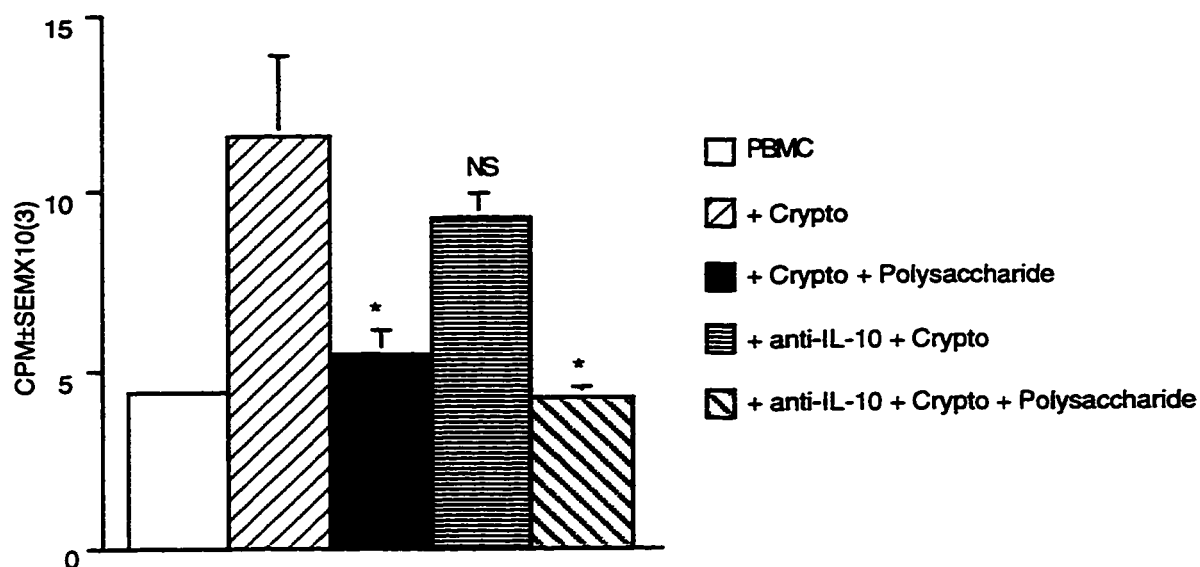


Figure 39: Treatment with anti-IL-10 does not augment lymphocyte proliferation abrogated by the presence of purified polysaccharide. PBMC (2×10^5 /well) were stimulated with *C. neoformans* (2×10^5 /well) in the presence or absence of polysaccharide ($50 \mu\text{g}/\text{ml}$) and/or anti-IL-10 ($10 \mu\text{g}/\text{ml}$). Thymidine incorporation was determined 7 days later. One of four representative experiments.

* $p < 0.05$ as compared to stimulated PBMC

NS - not significantly different as compared to stimulated PBMC

between T cells and APC, experiments were performed to determine if CPS was affecting the APC. PBMC or an acapsular strain of *C. neoformans* were incubated with CPS and the excess was removed by washing. To determine whether CPS suppresses lymphocyte proliferation by directly affecting the proliferating cells or by affecting the organism, PBMC or an acapsular strain of *C. neoformans* were incubated with purified polysaccharide and the excess was removed by washing before being used in culture. Preincubation of *C. neoformans* in CPS abrogated lymphocyte proliferation of untreated PBMC (Figure 40), while preincubation of PBMC did not effect lymphocyte proliferation in response to untreated *C. neoformans*. These results suggested that polysaccharide does not effect lymphocyte proliferation by affecting accessory molecules on cells, but rather was affecting the organism. To confirm that CPS was binding *C. neoformans*, treated organisms were examined by mucicarmine staining. Organisms possessing a capsule stain pink with mucicarmine, while acapsular organisms do not (Lazcano *et al.*, 1993). Polysaccharide treated organisms went from not staining to pink after incubation in polysaccharide (data not shown). Therefore, polysaccharide does not affect lymphocyte proliferation by directly affecting surface molecules on APC, but rather exerts its effect directly on the microorganism.

One of the major effects of the polysaccharide capsule is to inhibit phagocytosis. To determine whether impaired phagocytosis adversely affects lymphocyte proliferation, two approaches were used. *C. neoformans* or polysaccharide coated *C. neoformans* were put into culture with PBMC. The amount of phagocytosis was determined by

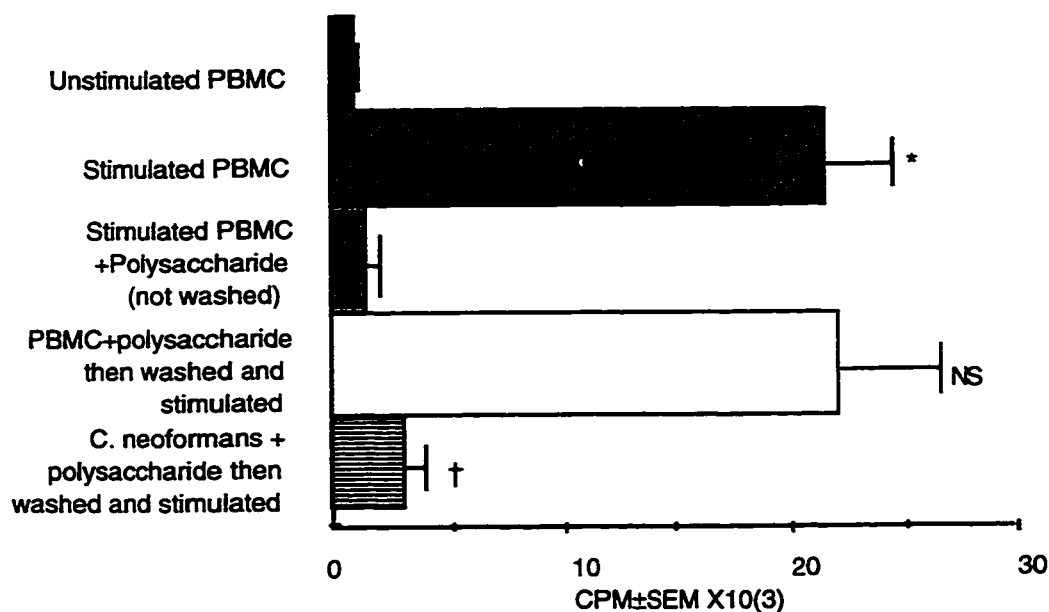


Figure 40: Lymphocyte proliferation in response to *C. neoformans* is abrogated when the organism is preincubated in purified polysaccharide. PBMC (2×10^5 /well) (■) and *C. neoformans* (2×10^5 /well) were cultured in the presence (▨) or absence (■) of $10 \mu\text{g/ml}$ purified polysaccharide. PBMC incubated for 1 hour at 37°C in purified polysaccharide then washed and co-cultured with untreated *C. neoformans* (□). *C. neoformans* was incubated for 1 hour at 37°C in purified polysaccharide then washed and co-cultured with untreated PBMC (▨). Lymphocyte proliferation was assessed seven days later by thymidine incorporation. * $p < 0.05$ as compared to unstimulated PBMC. NS - not significant compared to stimulated PBMC. † $p < 0.05$ compared to stimulated PBMC. One of four representative experiments.

giemsa staining and light microscopy, and lymphocyte proliferation was assessed by thymidine incorporation. Giemsa staining and light microscopy were previously found to be as reliable as FITC labeling for assessing uptake. Preincubation of *C. neoformans* in CPS decreased the amount of phagocytosis by 70%, which correlated with a reduction in lymphocyte proliferation (Figure 41). Increasing the effector to target cell ratio did not enhance uptake or lymphocyte proliferation of the polysaccharide treated organisms.

Having established that capsule polysaccharide could decrease phagocytosis and lymphocyte proliferation it was important to determine whether an independent factor that inhibited phagocytosis had an effect on lymphocyte proliferation. To examine the role of phagocytosis in the induction of lymphocyte proliferation and the generation of cell mediated immune response to *C. neoformans* organisms were put in culture in the presence of lidocaine (1-10000 μ M), which inhibits phagocytosis (Das and Misra, 1994). To ensure lidocaine treatment inhibited phagocytosis, uptake of *C. neoformans* was assessed after four hours in culture. A dose dependent inhibition in phagocytosis was observed (Figure 42A). This correlated with a dose dependent reduction in lymphocyte proliferation (Figure 42B). To ensure that the effects of lidocaine was not due to another mechanism, the response to a mitogen (concanavalin A) and superantigen (staphylococcal enterotoxin B) were tested in the presence of lidocaine. As expected lidocaine had no effect on proliferation to these stimuli (Figure 43; Berkeley *et al.*, 1994).

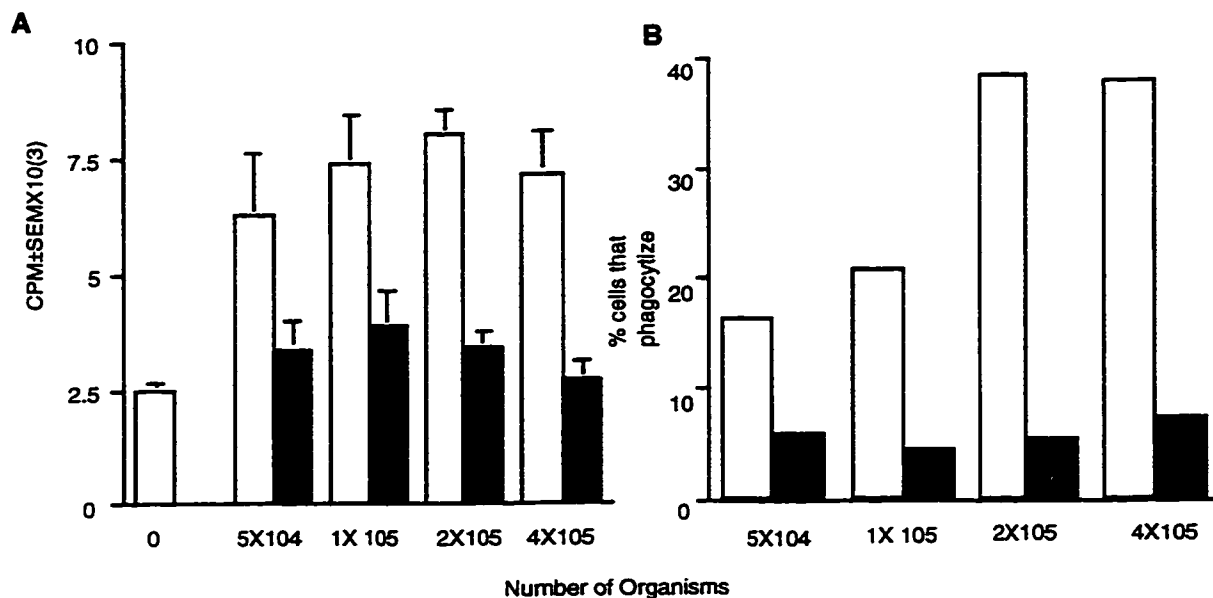


Figure 41: Preincubation of *C. neoformans* in purified polysaccharide reduced phagocytosis and decreased lymphocyte proliferation. PBMC (2×10^5 /well) were put into culture with various numbers of *C. neoformans* that had been left untreated (\square), or had been treated with purified polysaccharide (\blacksquare). After 7 days lymphocyte proliferation was assessed by thymidine incorporation (Panel A). In parallel (Panel B) adherent PBMC were put into culture with *C. neoformans* at the same effector to target cell ratio. 18 hours later coverslips were examined for the percentage of cells that had phagocytosed *C. neoformans*. One of three representative experiments.

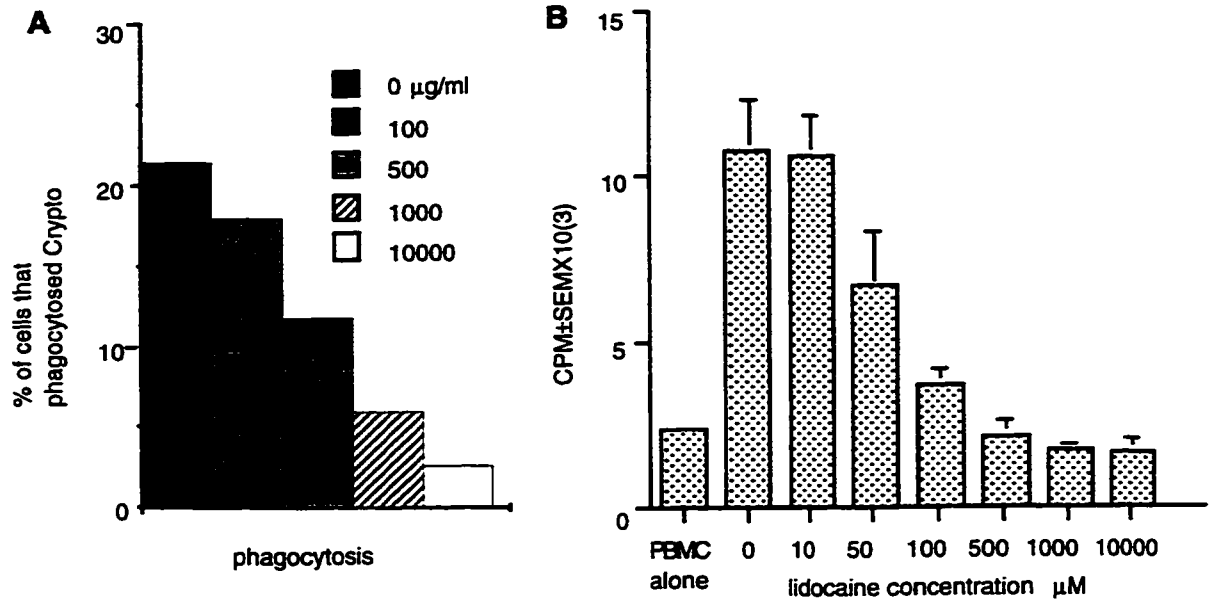


Figure 42A: Treatment with lidocaine inhibits uptake of *C. neoformans* in a dose dependent fashion. PBMC ($2 \times 10^5/\text{well}$) were stimulated with *C. neoformans* ($2 \times 10^5/\text{well}$) in the presence of various concentrations of lidocaine (0-10000 μM) 4 hours later uptake was determined by giemsa staining and light microscopy.

Figure 42B: Treatment with lidocaine inhibits lymphocyte proliferation in response to *C. neoformans*. PBMC ($2 \times 10^5/\text{well}$) were stimulated with *C. neoformans* ($2 \times 10^5/\text{well}$) in the presence of various concentrations of lidocaine (0-10000 μM). Seven days later thymidine incorporation was assessed. The experiment was performed 4 times with similar results.

One of four representative experiments.

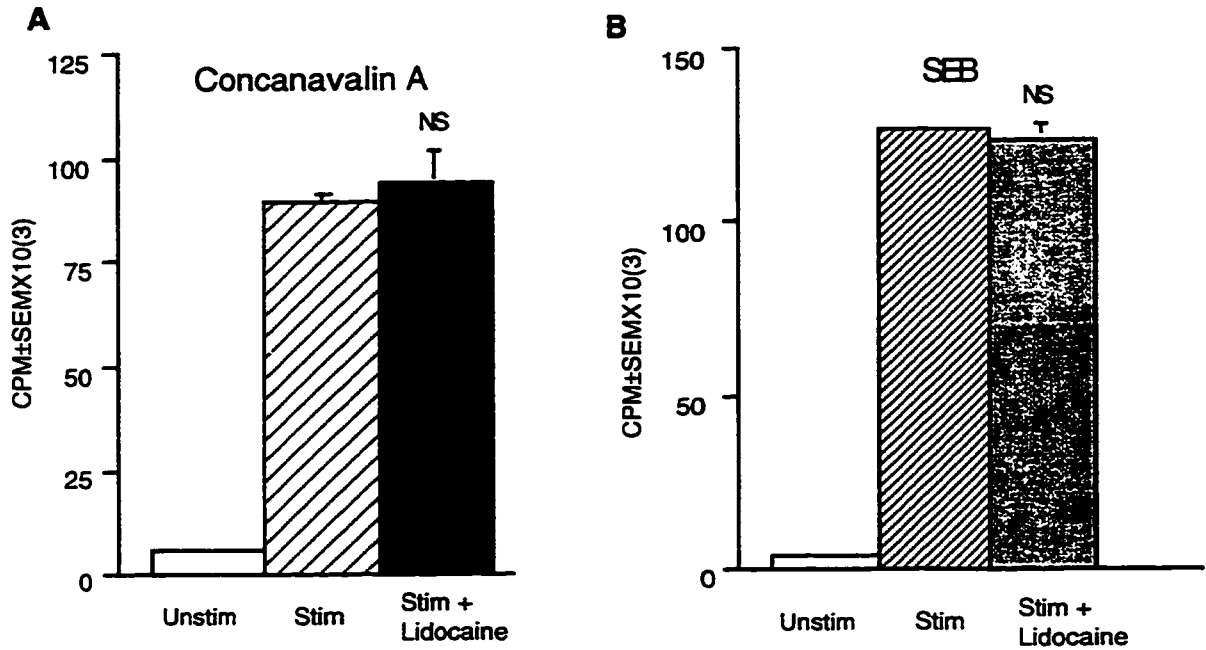


Figure 43: Incubation in the presence of lidocaine has no effect on lymphocyte proliferation to a mitogen or superantigen. PBMC (2×10^5 /well) were put into culture with $2.5 \mu\text{g/ml}$ Con A (Panel A) or $1 \mu\text{g/ml}$ SEB (Panel B) in the presence or absence of 10 mM lidocaine. Three or five days later lymphocyte proliferation was assessed by thymidine incorporation. One of four representative experiments. NS- not significantly different from cells stimulated in the absence of lidocaine.

6.2.4 Overcoming Suppression

Human AB serum

To determine if opsonization with human serum affected lymphocyte proliferation of *C. neoformans* that had been incubated in purified polysaccharide, coated *C. neoformans* was opsonized for 1 hour in human serum and then used in a proliferation assay. Opsonization with human serum augmented lymphocyte proliferation to polysaccharide treated *C. neoformans* (Figure 44A). This correlated with an increase in phagocytosis from 8% to 29%.

Anti-cryptococcal antibody

To determine if treatment with mAb could also overcome suppression of lymphocyte proliferation CPS treated *C. neoformans* were incubated with cryptococcal polysaccharide mAb (471) and then used to stimulate lymphocytes. Anti-CPS mAb significantly increased lymphocyte proliferation to polysaccharide treated *C. neoformans*, and correlated with an increase in phagocytosis from 10% to 45% (Figure 44B).

Opsonization of encapsulated strains of *C. neoformans*

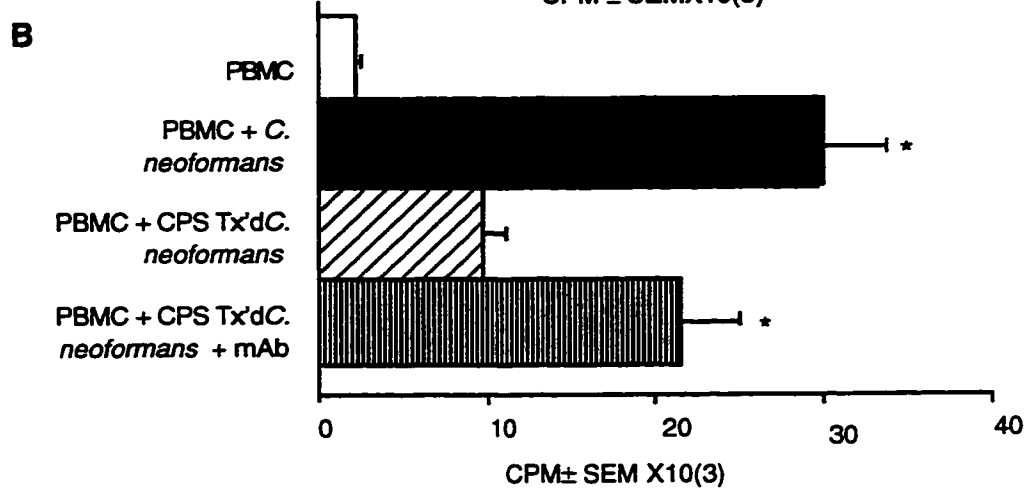
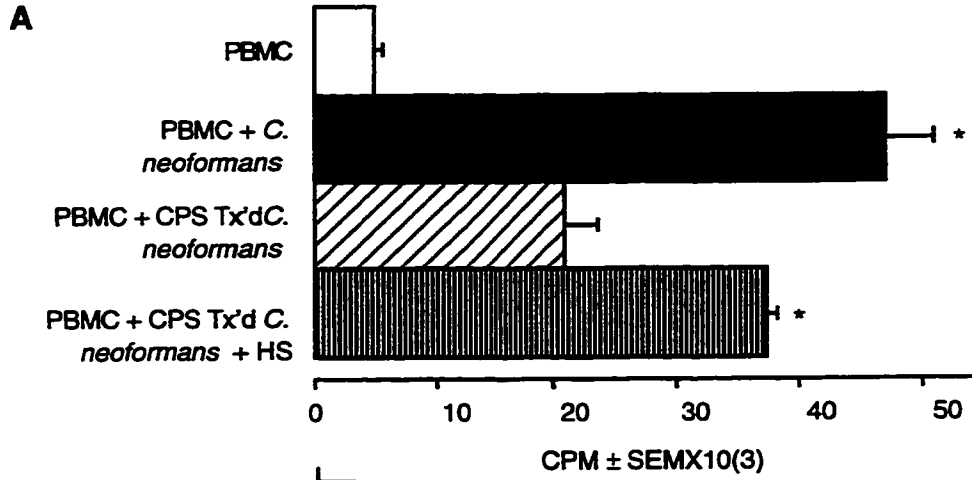
Having established the effects of free polysaccharide, experiments were performed to see if cell mediated responses to encapsulated strains of *C. neoformans* could be augmented. Pretreatment in normal human serum, or anti-capsular antibody increased

Figure 44A: Treatment with human serum augments lymphocyte proliferation to polysaccharide treated *C. neoformans*. PBMC (2×10^5 /well) (□) were stimulated with untreated *C. neoformans* (2×10^5 /well) (■), or *C. neoformans* (2×10^5 /well) that had been preincubated with 10 µg/ml purified polysaccharide (▨). Some polysaccharide treated organisms were then incubated with human serum (▩). Seven days later lymphocyte proliferation was assessed by thymidine incorporation.

* $p < 0.05$ as compared to control. The experiment was performed 5 times with similar results. One of five representative experiments.

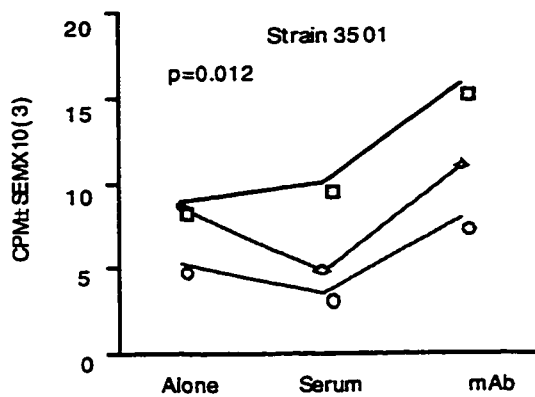
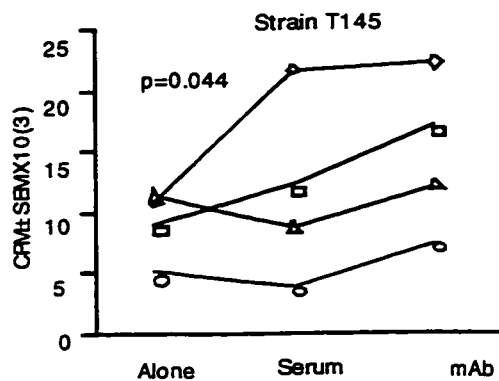
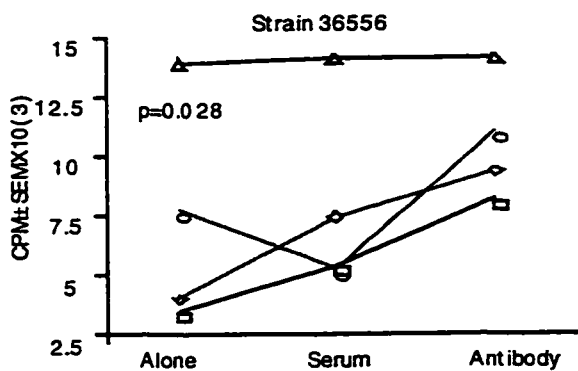
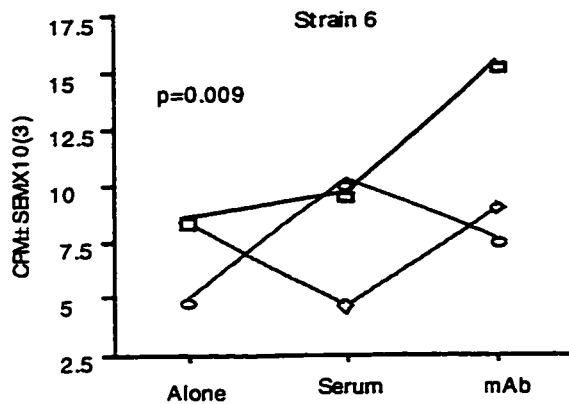
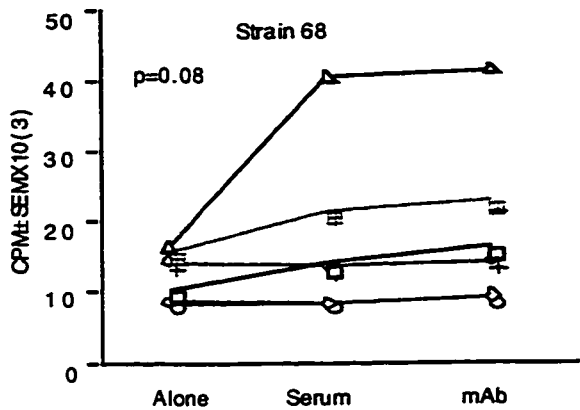
Figure 44B: Treatment with antipolysaccharide monoclonal antibody augments lymphocyte proliferation to polysaccharide treated *C. neoformans*. PBMC (2×10^5 /well) (□) were stimulated with untreated *C. neoformans* (2×10^5 /well) (■), or *C. neoformans* (2×10^5 /well) that had been preincubated with 10 µg/ml purified polysaccharide (▨). Some polysaccharide treated organisms were then incubated with mAb to polysaccharide (▩). Seven days later lymphocyte proliferation was assessed by thymidine incorporation.

* $p < 0.05$ as compared to control. One of five representative experiments.



lymphocyte proliferative responses to several encapsulated strains of *C. neoformans*. This was true for strains of serotype A and D. Treatment with an anti-CPS antibody was significantly better than human serum at augmenting lymphocyte proliferation (Figure 45).

Figure 45: Anti-capsular antibody is better than human serum for augmenting T cell responses. Various encapsulated strains of *C. neoformans* (68, 6, 36556, T145 and 3501) were left alone, or pretreated with anti-CPS antibody or normal human serum for 1 hour at 37°C. The organisms were washed and put into culture at 2×10^5 /well with PBMC (2×10^5 /well). Seven days later lymphocyte proliferation was assessed by thymidine incorporation. Each line represents an individual experiment proliferation. Paired t tests were performed for each strain.



6.3 DISCUSSION

C. neoformans has many virulence factors including polysaccharide capsule, and serotype, as both have correlates to infection (Dykstra *et al.*, 1977; Bennett *et al.*, 1977; Fromtling *et al.*, 1982; Cherniak and Sundstrom, 1994). It was likely that they would have an effect on cell mediated immunity. In this chapter these two components of virulence were examined for their ability to alter lymphocyte proliferation.

6.3.1 Serotype

C. neoformans var. neoformans consists of two serotypes, A and D. These serotypes differ in the degree of mannosyl substitution and molar ratios of xylose, mannose and glucuronic acid, but have a common core (Kozel *et al.*, 1988a). Studies in both HIV+ and HIV- individuals have shown that they are predominately infected with serotype A organisms (Mitchell and Perfect, 1995). In fact, upon the advent of AIDS serotype patterns changed so that serotype A became the predominate serotype reported worldwide, even in areas endemic for *C. neoformans var. gatti* (Sukroongreung *et al.*, 1996). Although individual and environmental factors could be associated with the serotype of the infecting strain of *C. neoformans*, the majority of cases of cryptococcosis are still attributed to serotype type A (Dromer *et al.*, 1996b). The ability of strains of *C. neoformans*, from the two major serotypes, to stimulate lymphocytes to proliferate was examined. Previous studies demonstrated that human lymphocytes proliferate in

response to serotype A organisms (Miller and Puck, 1984). It was not known; however, whether the response of lymphocytes was unique to this serotype of *C. neoformans*. Since the majority of cryptococcal infections are produced by serotype A organisms (Bennett *et al.*, 1977) it is possible that serotype D *C. neoformans* elicit a greater lymphocyte response, and this is one reason they are less common.

Organisms of both serotype A and D were found to be capable of eliciting lymphocyte proliferative responses. The degree of lymphocyte proliferation was similar following stimulation by clinical isolates of serotypes A and D, which had comparable capsule sizes. These data suggests that the antigen responsible for lymphocyte proliferation is not present uniquely on serotype A organisms. Since strains of either serotype and acapsular mutants can stimulate lymphocytes to proliferate, this also suggests that the antigen that is responsible for serotype is not the one responsible for proliferation. This is not an unexpected finding as the responding lymphocytes are T cells (Miller and Puck, 1984; Chapter 3). A T cell response implies that the antigen is a protein, while serotype antigens are polysaccharides. Because, the proliferative response was similar with either serotype the lymphocyte response does not explain the advantage of one serotype over another to produce cryptococcosis.

6.3.2 Polysaccharide Capsule

One of the most important virulence factors of *C. neoformans* is its polysaccharide capsule. The capsule is synthesized upon entry of the organism into the

host (Farhi *et al.*, 1970). The capsule can vary quite dramatically in size becoming as large as 30 μm in diameter (Perfect, 1989; Levitz, 1991a).

Initial experiments investigating polysaccharide capsule found that strains of *C. neoformans* that have a small polysaccharide capsule stimulate lymphocytes to proliferate to a greater extent than those that have a large capsule. The size of the polysaccharide capsule is a determinant of the virulence of *C. neoformans* (Mitchell and Friedman, 1972; Dykstra *et al.*, 1977; Fromtling *et al.*, 1982). Although cryptococcal capsular polysaccharide attenuates the lymphocyte response in mice (Collins and Bancroft, 1991; Murphy and Cozad, 1972), capsular polysaccharide has not previously been shown to attenuate the lymphocyte response in humans. The lymphocyte response to an acapsular strain was not suppressed by organisms that have a large capsule, and addition of purified capsular polysaccharide suppressed the response.

These studies suggest that the size of the capsule might contribute to virulence by suppressing lymphocyte proliferation. *C. neoformans* is a frequent fungal pathogen in AIDS patients, and infections occur with strains that have a small polysaccharide capsule (Bottone *et al.*, 1986). By contrast, isolates of *C. neoformans* from non-AIDS patients are seldom minimally encapsulated. Together these data demonstrates that minimally encapsulated organisms stimulate a brisk lymphocyte response, and would predict that infections would only occur due to minimally encapsulated organisms in circumstances where there was profound lymphocyte immunosuppression.

The size of the cryptococcal capsule increases when *C. neoformans* enters the physiologic conditions of the host during an infection (Granger *et al.*, 1985). Organisms possessing a large capsule; however, do not suppress the response to organisms that are minimally encapsulated. This observation suggests that organisms that have responded to the physiologic stimuli, and begun to synthesize larger amounts of capsule would not be able to suppress the response to organisms that had not yet begun to synthesize large amounts of capsule. Exogenous or “free” CPS; however, inhibits responses to an acapsular strain. This would suggest that free capsular polysaccharide, perhaps from killed organisms, is capable of suppressing the lymphocyte response. In sum, these investigations confirm murine studies that capsule size correlates with lymphocyte proliferation (Collins and Bancroft, 1991), and shows that exogenous capsule can inhibit lymphocyte proliferative response to an acapsular strain. The mechanism by which CPS suppresses lymphocyte proliferation is unknown.

Mechanism of Inhibition

Three observations were made regarding CPS induced suppression of lymphocyte proliferation 1) CPS did not inhibit lymphocyte proliferation by causing production of IL-10, or by directly affecting antigen presenting or accessory cells 2) CPS suppressed phagocytosis and this correlated with lymphocyte proliferation 3) monoclonal anti-capsular antibody increased lymphocyte proliferation and phagocytosis, and was more effective than human serum.

Cryptococcal capsular polysaccharide (CPS) causes a multitude of effects. It can induce the release of proinflammatory (Retini *et al.*, 1996) and immunosuppressive cytokines (Vecchiarelli *et al.*, 1996). CPS can also inhibit TNF- α and IL-1 β production (Vecchiarelli *et al.*, 1995) which are important in host defense to *C. neoformans* (Blasi *et al.*, 1995; Huffnagle *et al.*, 1996). Further, CPS can also effect leukocyte infiltration in inflammatory responses by causing shedding of L-selectin and TNF- α R from neutrophils (Dong and Murphy, 1996). These effects all suggest possible mechanisms by which CPS could abrogate lymphocyte proliferation.

IL-10 is an immunosuppressive cytokine. It has been reported to reduce antigen specific T cell responses (de Waal Malefyt *et al.*, 1991; Del Prete *et al.*, 1993). This is believed that to occur by reducing the antigen presenting capacity of monocytes (de Waal Malefyt *et al.*, 1991; Monari *et al.*, 1997) by downregulating the expression of HLA-DR on monocytes (Monari *et al.*, 1997; Retini *et al.*, 1998), but causes a significant increase in expression on dendritic cells (Morel *et al.*, 1997). Although IL-10 production can be induced by CPS (Vecchiarelli *et al.*, 1996), it was not responsible for the observed reduction in lymphocyte proliferation. This finding is in contrast to recent studies by Retini and colleagues (1998), who found that addition of anti-IL-10 to cultures with encapsulated *C. neoformans* restored proliferation to levels of acapsular strains. These differences can be explained by differences in experimental design. In the studies by Retini and colleagues (1998) responses of an encapsulated and acapsular strain of *C. neoformans* were compared in the presence and absence of anti-IL-10. These strains were not isogenic

so the effect they observed may relate to phenotypic differences of the strains. It may also be that the amount of polysaccharide in our cultures overwhelmed the anti-IL-10 effect, although this seems unlikely based upon the low levels of IL-10 that are secreted in response to CPS (P. Warren, personnel communication).

CPS can also effect surface molecule expression including adhesion molecules (Dong and Murphy, 1996) and DR expression (Monari *et al.*, 1997; Retini *et al.*, 1998) on neutrophils and monocytes respectively. By determining whether CPS affected host cells, or the organism it was determined that CPS was not interfering with accessory functions. Retini and colleagues (1998) speculate that CPS binds to monocytes to modulate induction of IL-10 release. The studies present here demonstrate that CPS has no inhibitory effect on lymphocyte proliferation unless it is incubated with the organism.

Binding of free polysaccharide to cryptococcal organisms has been well described (Kozel and Harmerath, 1984; Small and Mitchell, 1986). Incubation of nonencapsulated *Cryptococcus* with purified cryptococcal polysaccharide renders nonencapsulated cells resistant to phagocytosis (Bulmer and Sans, 1968; Bulmer and Tacker, 1975; Kozel and Mastroianni, 1976; Kozel, 1977; Kozel and McGaw, 1979; Small and Mitchell, 1989). It was determined that CPS bound to acapsular *C. neoformans* limited binding and uptake and suppressed lymphocyte proliferation. Because systemic cryptococcosis is characterized by high titers of cryptococcal polysaccharide in serum (Diamond and Bennett, 1974), this free polysaccharide may bind to organisms and further inhibit their uptake, and ultimately affect the induction of cell mediated immune responses. The

importance of phagocytosis for lymphocyte proliferation was further confirmed by the inhibitory effect of lidocaine.

Over coming Inhibitory Effects of CPS

Human Serum

Knowing that diminished lymphocyte proliferation in the presence of CPS was due to a decrease in uptake, it was of interest to determine whether opsonizing the organisms might increase uptake and restore lymphocyte proliferation. Initially, human sera was used as a source of opsonins. Opsonic fragments for *C. neoformans* in normal human serum include C3 and anti-cell wall glucan antibody (Kozel and McGaw, 1979; Levitz and Dibenedetto, 1988; Levitz *et al.*, 1993). Capsule can allow for deposition of these fragments on the surface of the yeast which would aid in uptake (Kozel *et al.*, 1984). Human serum with active complement can opsonize *C. neoformans* while heat inactivated sera does not (Davies *et al.*, 1982). Both encapsulated and acapsular strains of *C. neoformans* activate the complement system (Diamond and Erikson, 1982; Laxalt and Kozel, 1979), but encapsulated strains are more potent activators than acapsular strains (Young and Kozel, 1993). This activation leads to deposition of C3 fragments on the capsule. Encapsulated organisms are opsonized by C3 fragments which bind to the capsule (Levitz and Dibenedetto, 1988; Levitz and Farrell, 1990; Levitz and Tabuni, 1991). In a murine system opsonization in serum increases lymphocyte proliferative

responses to encapsulated and acapsular strains of *C. neoformans* (Collins and Bancroft, 1991). Opsonization in human serum was found to augment lymphocyte proliferation to polysaccharide treated acapsular, and encapsulated strains of *C. neoformans*.

Anti-cryptococcal Antibody

The role of antibody in protection from cryptococcosis is controversial. Patients that are predisposed to cryptococcal infections have defects in cell mediated immunity. By contrast, patients with isolated defects in antibody production do not have a meaningful increase in the incidence of cryptococcal infections. This has led to the assumption that T cell-mediated immunity is important, while humoral immunity is not. There are, however, numerous studies that demonstrate that anticryptococcal antibody is protective in murine models (Dromer *et al.*, 1989; Mukherjee *et al.*, 1994; Yuan *et al.*, 1995; Zhong and Pirofski, 1998). Further, AIDS patients have lower levels of anti-CPS antibody than healthy adults (Dromer *et al.*, 1988), and there is also a relationship between a favorable outcome and detectable antibody during infection (Diamond and Bennett, 1974).

Cryptococcal anti-capsular antibodies can be quite variable in their ability to confer protection. They can be protective, nonprotective or disease enhancing antibodies depending on isotype and epitope specificity (Mukherjee *et al.*, 1994; Mukherjee *et al.*, 1995; Yuan *et al.*, 1995). This suggests that anticryptococcal antibodies have multiple

effects on the immune response, and may explain the variable results obtained with polyclonal antibody preparations.

The success of immunization (vaccination or passive) is based upon the capacity of human antibodies against cryptococcal capsular polysaccharide to enhance effector cell function (Mozaffarian *et al.*, 1995; Mukherjee *et al.*, 1995). This has traditionally focused on antifungal activity rather than on elicitation of cell mediated immunity. Protective antibody administration results in a decrease in fungal burden, prolonged survival, and decreased in polysaccharide antigen in the serum (Pirofski and Casadevall, 1996). More recently presence of anti-capsular antibody has been found to increase production of IL-1 β , TNF- α and IL-2, while decreasing IL-10 secretion (Vecchiarelli *et al.*, 1998b). Since T cells are clearly important in cryptococcal host defense the possibility that protective antibody might somehow influence T cell responses and enhance host defense by this mechanism was considered. This hypothesis is supported by recent studies in which Yuan and colleagues (1997) have demonstrated that T cells can cooperate with administered antibody in cryptococcal infections to induce protective responses and increase survival in a murine model; and studies by Vecchiarelli and colleagues (1998b) where anticryptococcal antibody could augment lymphocyte proliferation.

An antibody was selected that had been demonstrated to be protective. It was found that opsonization of polysaccharide treated organisms, or encapsulated strains of *C. neoformans* with anti-capsular antibody augment lymphocyte proliferation. There are

a number of mechanisms by which antibody might enhance cell mediated immune responses. Anti-cryptococcal IgG antibody can promote antibody dependent cellular cytotoxicity (Mukherjee *et al.*, 1995), or it could block the mechanism by which CPS induces immunosuppressive cytokines (Vecchiarelli *et al.*, 1998b). The studies presented here indicate that one mechanism by which anti-capsular antibody (or human serum) enhances cell mediated immunity is to promote the uptake of organisms. This suggests that anti-capsular antibody may enhance host defense at least in part by improving presentation of *C. neoformans* to T cells which would promote cell mediated immune responses.

Opsonization of *C. neoformans* with components of complement or anti-capsular antibody could allow dendritic cells, which express FcR and CR, to increase uptake and ultimately augment proliferative responses. Such opsonization also augments uptake by other phagocytic cells (Kozel *et al.*, 1984; Zhong and Pirofski, 1996; Cross *et al.*, 1997). Increased killing could lead to antigenic debris that dendritic cells could take up and present, again enhancing proliferative responses.

Final experiments were performed to determine whether opsonization with monoclonal anticapsular antibody alone was as good, or better than human sera. Lymphocyte proliferation in the presence of a specific anticapsular antibody was found to be marginally, although statistically superior to human sera.

The studies presented here also provide a rationale for vaccine therapy for cryptococcosis. A vaccine has been prepared by conjugating GXM to tetanus toxoid

(Devi *et al.*, 1991). This vaccine elicits a murine and human antibody response (Devi *et al.*, 1991; Pirofski, *et al.*, 1995; Devi, 1996). Antibodies to glucuronoxylmannan conjugated to tetanus toxoid promote phagocytosis of *C. neoformans* in the absence of complement (Zhong and Pirofski, 1996), and the vaccine is protective in a murine model (Devi, 1996). It may be that one of the mechanisms by which these antibodies are effective is by enhancing the uptake of organisms by antigen presenting cells, and thus enhancing presentation to T cells with subsequent optimal cell mediated immunity.

In summary, exogenous polysaccharide can inhibit cell mediated immune responses by binding to organisms and reducing their uptake by antigen presenting cells. Further, opsonization by normal human serum and by anti-capsular antibody can augment the responses to *C. neoformans*. Thus, there is the therapeutic potential of stimulating cell mediated immunity with anticapsular antibody.

CHAPTER SEVEN

CONCLUSIONS

Cryptococcus neoformans is a pathogenic encapsulated yeast that causes devastating infections in AIDS patients. A model of the induction of cell mediated immune responses to this pathogen can be developed based upon what is known about *C. neoformans* and the findings in this thesis (Figure 46).

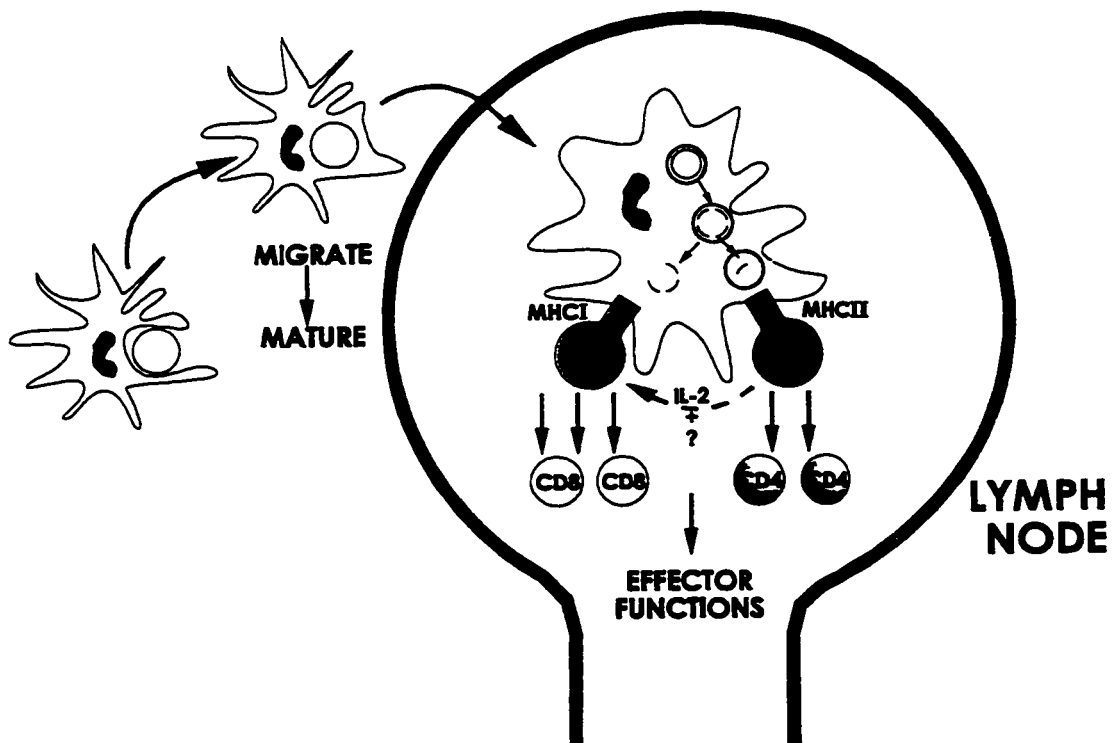
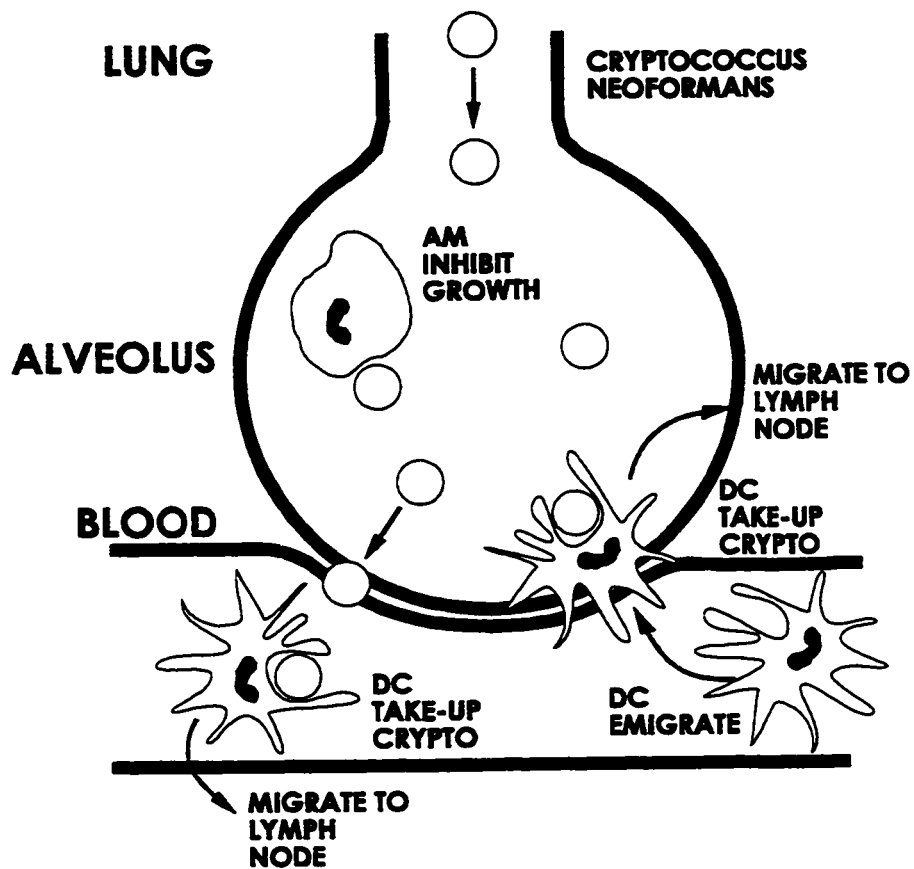
Acapsular, asexual *C. neoformans* is acquired by the respiratory route as an aerosol. Here the organisms come into contact with resident alveolar macrophages. Alveolar macrophages (AM) can inhibit growth of the fungus (Weinberg *et al.*, 1987), but only have poor killing abilities unless they are activated (Mody *et al.*, 1991). These cells take up *C. neoformans* and if they come into contact with a T cell in the alveolus may induce a response. As alveolar macrophages do not migrate to regional compartments (Havenith *et al.*, 1993a; 1993b), it is unlikely they have a significant role in generating cell mediated immunity. Next one of two things can happen. *C. neoformans* can penetrate into the interstium of the lung then into the blood and contact dendritic cells (DC) here, or dendritic cells could emigrate from the blood into the airspace. Dendritic cells have been documented to very rapidly emigrate to the airway in response to inhaled bacteria, inflammatory stimuli or mediators (McWilliams *et al.*, 1994; Lambrecht *et al.*, 1996). Once *C. neoformans* contacts dendritic cells it is actively taken up, likely via the mannose

Figure 46: Model of development of a cell mediated immune response to *C. neoformans*.

AM - alveolar macrophage

DC - dendritic cell

Crypto - *C. neoformans*



receptor (Chapter 4). As this occurs the organisms begins to synthesize polysaccharide (Farhi *et al.*, 1970; Granger *et al.*, 1985) which inhibits uptake and leads to increased virulence of the organism (Chapter 6). Once dendritic cells have ingested the organism they migrate to secondary lymphoid organs via the lymphatics or the blood. During this process they mature and acquire an immunostimulatory phenotype. A debate is ongoing as to whether dendritic cells take up and process antigen and differentiate as they migrate to regional compartments (such as lymph nodes), or whether they take up the organism begin processing, migrate to the regional compartments continue processing and then differentiate. It seems likely that the later occurs as dendritic cells regulate their processing capabilities, and are known to retain antigen in MIIC vesicles for days at a time (Norbury *et al.*, 1995). Successful processing of the organism is dependent upon the activity of lysosomal proteases, such as cathepsin B and D (Chapter 5). Once within secondary lymphoid organs the dendritic cells are able to present cryptococcal antigen to T cells (Chapter 4).

Both class I and II MHC molecules are used to initiate T cell responses (Chapter 3). Class I presentation may be the result of the organism escaping into the cytosol, or antigen “leakage” into the cytosol for interaction with MHC I molecules. Both CD4 and CD8 cells activated and proliferate in response to *C. neoformans*, as they express CD69, early cyclins of the cell cycle, growth factor receptors and increase in number (Chapter 3). CD8 cells however; are dependent upon the presence of CD4 cells, or a CD4 derived signal to continue through the cell cycle, express T cell growth factor receptors and

proliferate. This signal is likely to involve another signal apart from IL-2 as the addition of this cytokine has been shown to be inadequate to push CD8 cells through the cell cycle and onto proliferate. The importance of CD4 and CD8 cells in the afferent phase of cell mediated immunity is well documented in murine models (Mody *et al.*, 1990; Hill and Harmsen, 1991; Huffnagle *et al.*, 1991a). The role of human CD4 and CD8 cells is less well defined, but they very likely have similar functions. These activated T cells will proliferate, release cytokines, and initiate a variety of effector functions, such as activating alveolar macrophages.

Based upon this model the possibility of exploiting dendritic cells as adjuvants can be imagined. Blood dendritic cells could be “loaded” with killed acapsular *C. neoformans*. This would allow for increased presentation, and would avoid the suppressive effects of polysaccharide or any risk of infection. Productive epitopes would be generated and protection would result. In uninfected individuals this protocol could be used to induce protective responses. During infection the same protocol could be used to stimulate a T cell response when *Cryptococcus* might be sequestered in granulomas, or had begun to synthesize capsule and suppress lymphocyte proliferation.

A second therapeutic possibility exists once the nature of the CD4 dependent signal for CD8 cell proliferation is uncovered. Administration of this “factor” could allow for CD8 responses to occur.

A final therapeutic possibility is directed toward the suppressive polysaccharide capsule. Cryptococcal polysaccharide, which the organism begins to synthesize shortly

after entering the lung, decreases cell mediated immune responses by reducing uptake of the organism. This suppression can be overcome *in vitro* by opsonization with anti-cryptococcal antibodies (Chapter 6). Thus, there is the potential to enhance cell mediated responses by therapy with anti-cryptococcal antibodies.

REFERENCES

1. Adema G.J. F. Hargers, R. Verstraten, E. de Vries, G. Marland, S. Menon, J. Foster, Y. Xu, P. Nooyen, T. McClanahan, K.B. Bacon, and C.G. Figdor. 1997. A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature*. 387:713-717.
2. Aguirre K., E.A. Havell, G.W. Gibson, and L.L. Johnson. 1995. Role of tumor necrosis factor and gamma interferon in acquired resistance to *Cryptococcus neoformans* in the central nervous system of mice. *Infect. Immun.* 63(5):1725-1731.
3. Allen, P. M., B. P. Babbitt, and E. R. Unanue. 1987. T cell recognition of lysozyme: The biochemical basis of presentation. *Immunol. Rev.* 98:171-187.
4. Alpuche-Aranda C.M., E.L. Racoosin, J.A. Swanson, and S.I. Miller. 1994. *Salmonella* stimulate macrophage macropinocytosis and persist within spacious phagosomes. *J. Exp. Med.* 179(2):601-608.
5. Amigorena S. and C. Bonnerot. 1998. Role of B-cell and Fc receptors in the selection of T-cell epitopes. *Curr. Opin. Immunol.* 10:88-92.
6. Armstrong D. 1988. Life threatening opportunistic fungal infection in patients with the acquired immunodeficiency syndrome. *Ann, NY.* 544:443-450.
7. Austyn J.M. 1993. Dendritic cells in transplantation. *Adv. Exp. Med. Biol.* 329:489-494.
8. Avrameas A., D. Mclroy, A. Hosmalin, B. Autran, P. Debre, M. Monsigny, A. C. Roche, and P. Midoux. 1996. Expression of a mannose/fucose membrane lectin on human dendritic cells. *Eur. J. Immunol.* 26:394-400.
9. Banchereau J. and R.M. Steinman. Dendritic cells and the control of immunity. *Nature*. 292:245-2252.
10. Barbey C., C. Watts, G. Corradin. 1995. Antigen-processing organelles from DRB1*1101 and DRB1*1104 B cell lines display a differential degradation activity. *Eur. J. Immunol.* 25:30-36.

11. Bauer A Rutenfrans I, Kirchner H. 1988. Processing requirements for T cell activation by *Mycoplasma arthritidis* derived mitogen. Eur. J. Immunol. 13:2109-2112.
12. Bava A. J. and R. Negroni. 1992. The epidemiological characteristics of 105 cases of cryptococcosis diagnosed in the republic of Argentina between 1981-1990. Rev. Inst. Med. Trop. Sao Paulo. 34(3):335-340..
13. Bennett, J. E., K. J. Kwon-Chung, and D. H. Howard. 1977. Epidemiologic differences among serotypes of *Cryptococcus neoformans*. Am. J. Epidemiol. 105:582-6.
14. Bennett, J.E., W.E. Dismukes, R.J. Duma, G. Medoff, MA. Sande, H. Gallis, J. Leonard, BT Fields, M. Bradshaw, H. Haywood, Z.A. McGee, T.R. Cate, C.G. Cobbs, J.F. Warner, and D.W. Alling. 1979. A comparison of amphotericin B alone and combined with flucytosine in the treatment of cryptococcal meningitis. N. Engl. J. Med 301:126-131.
15. Berkeley M.B., S. Daussin, M.C. Hernandez, and B.M. Bayer. 1994. *In vitro* effects of cocaine, lidocaine and monoamine uptake inhibitors on lymphocyte proliferative responses. Immunopharm Immunotoxicol. 16(2):165-178.
16. Berzofsky J.A., S.J. Brett, H.Z. Streicher, and H. Takahashi. 1988. Antigen processing for presentation to T lymphocytes: function, mechanisms, and implications for the T-cell repertoire. Immunol. Rev. 106:5-31.
17. Bhardwaj N., J.W. Young, A. J. Nisanian, J. Baggers, and R. M. Steinman. 1993. Small amounts of superantigen, when presented on dendritic cells, are sufficient to initiate T cell responses. J. Exp. Med. 178:633-642.
18. Bhattacharjee A.K., J.E. Bennett, and C.P.J. Glaudemans. 1984. Capsular polysaccharides of *Cryptococcus neoformans*. Rev. Infect. Dis. 6:619-624.
19. Bianco C., F.M. Griffin, and S.C. Silverstein. 1975. Studies of the macrophage complement receptor. Alternation of receptor function upon macrophage activation. J. Exp. Med. 141:1278-1290.
20. Biselli, R., P. M. Matricardi, R. DAmelio, and A. Fattorossi. 1992. Multiparametric flow cytometric analysis of the kinetics of surface molecule expression after polyclonal activation of human peripheral blood T lymphocytes. Scand. J. Immunol. 35:439-447.
21. Blackstock R., J.M. McCormack and N.K. Hall. 1987. Induction of a macrophage suppressive lymphokine by soluble cryptococcal antigens and its association with models of immunological tolerance. infect. Immun. 55: 233-239.

22. Blasi E., R. Barluzzi, R. Mazzolla, L. Pitzurra, M. Puiti, S. Saleppico and F. Bistoni. 1995. Biomolecular events involved in anticryptococcal resistance in the brain. *Infect. Immun.* 63(4):1218-1222.
23. Bonnerot C., D. Lankar, D. Hanau, D. Spehner, J. Davoust, J. Salamero, and W.H. Fridman. 1995. Role of B cell receptor Ig alpha and Ig beta subunits in MHC class II-restricted antigen presentation. *Immunity.* 3:335-347.
24. Bottone, E. J., M. Toma, and B. E. Johansson. 1986. Poorly encapsulated *Cryptococcus neoformans* from patients with AIDS: I: Preliminary observations. *AIDS Res.* 2:211-8.
25. Bozzette S.A., R. Larsen, J. Chiu et al. 1991. A controlled trial of maintenance therapy with fluconazole after treatment of cryptococcal meningitis in the acquired immunodeficiency syndrome. *N. Engl. J. Med.* 324:580-584. fluconazole therapy
26. Breen, J. F., I. C. Lee, F. R. Vogel, and Friedman-H. 1982. Cryptococcal capsular polysaccharide-induced modulation of murine immune responses. *Infect. Immun.* 36:47-51.
27. Brown E.J. 1995. Phagocytosis. *BioEssays.* 17:109-117.
28. Bulmer G. S., and J. R. Tacker. 1975. Phagocytosis of *Cryptococcus neoformans* by alveolar macrophages. *Infect. Immun.* 11:73-9.
29. Bulmer G.S. and M.D. Sans. 1968. *Cryptococcus neoformans*. II. Phagocytosis by human leukocytes. *J. Bacteriol.* 94:1480-1483.
30. Buschke A. 1895. Über eine durch Coccidien Hervergerufene Krankheit des menschen. *Dtsch. Med. Wochenschr.* 21:14.
31. Busse O. 1895. Über Parasotare zelleinschlusse und ihre zuchtung. *Zentralbl. Bakteriol.* 16:175-180.
32. Buus S. And O. Wedelin. 1986. A group-selective inhibitor of lysosomal cysteine proteinases selectively inhibits both proteolytic degradation and presentation of the antigen dinitrophenyl-ploy-L-lysine by guinea pig accessory cells. *J. Immunol.* 135:452-458.
33. Cantrell, D. A. and K. A. Smith. 1984. The interleukin-2 T-cell system: A new cell growth model. *Science* 224:1312-1316.
34. Cardell, S., B. Sander, and G. Moller. 1991. Helper interleukins are produced by both CD4 and CD8 splenic T cells after mitogen stimulation. *Eur. J. Immunol.* 21:2495-2500.

35. Carrera, A. C., F. Sanchez-Madrid, M. Lopez-Botet, C. Bernabeu and De-Landazuri-M-O. 1987. Involvement of the CD4 molecule in a post-activation event on T cell proliferation. *Eur. J. Immunol* 17:179-186.
36. Castellino F. and R.N. Germain. 1995. Extensive trafficking of MHC class II invariant chain complexes in the endocytic pathway and appearance of peptide-loaded class II in multiple compartments. *Immunity*. 2:73-88.
37. Caux C., B. Vanbervliet, C. Massacrier, M. Azuma, K. Okumura, L.L. Lanier, and J. Banchereau. 1994. B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *J. Exp. Med.* 180:1841-1847.
38. Cebrian, M., E. Yague, M. Rincon, M. Lopez-Botet, M. O. de Landazuri and F. Sanchez-Madrid. 1988. Triggering of T cell proliferation through AIM, an activation inducer molecule expressed on activated human lymphocytes. *J. Exp. Med.* 168:1621-1637.
39. Cella M., A. Engering, V. Pinet, J. Pieters, and A. Lanzavecchia. 1997. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature*. 388:782-787.
40. Chain B.M., and P.M. Kaye. 1986. Processing and presentation by dendritic cells - the role of the lysosome in antigen breakdown. In: *Regulation of immune gene expression*. eds. M. Feldmann, and A. McMichael. Humana Press, New Jersey. p. 207.
41. Chain B.M., P.M. Kaye and M. Shaw. 1988. The biochemistry and cell biology of antigen processing. *Immunol. Rev.* 106:33-58.
42. Chaka W., A.F. Verheul, V.V. Vaishnav, R. Cherniak, J. Scharringa, J. Verhoef, H. Snippe, and I.M. Hoepelman, 1997. *Cryptococcus neoformans* and cryptococcal glucuronoxylomannan, galactoxylomannan, and mannoprotein induce different levels of tumor necrosis factor alpha in human peripheral blood mononuclear cells. *Infect. Immun.* 65:272-278.
43. Chaka W., J. Scharringa, A.F. Verheul, J. Verhoef, A.G. Van Strijp, and I.M. Hoepelman. 1995. Quantitative analysis of phagocytosis and killing of *Cryptococcus neoformans* by human peripheral blood mononuclear cells by flow cytometry. *Clin. Lab. Immunol.* 2(6):753-759.
44. Chapman H.A. 1998. Endosomal proteolysis and MHC class II function. *Curr. Opin. Immunol.* 10:93-102.

45. Chen G.H., J.L. Curtis, C.H. Mody, P.J. Christensen, L.R. Armstrong, and G.B. Toews. 1994. Effect of granulocyte-macrophage colony-stimulating factor on rat alveolar macrophage anticryptococcal activity *in vitro*. *J. Immunol.* 152(2):724-734.
46. Cherniak, R., and J.B. Sundstrom. 1994. Polysaccharide antigens of the capsule of *Cryptococcus neoformans*. *Infect. Immun.* 62:1507-1512.
47. Chestnut, RAW., and H. M. Gray. 1986. Antigen presentation by B cells and its significance in T-B Interaction. *Adv. Immunol.* 39:51-94.
48. Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* 162:156-159.
49. Chuck S. L. and M. A. Sande. 1989. Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. *N. Engl. J. Med.* 321:794-799.
50. Clumeck N., M. Carael, and P. Van de Perre. 1989. The African AIDS experiment in contrast with the rest of the world. In: Leoung G. Mills J eds. *Opportunistic infections in patients with the acquired immune deficiency syndrome*. New York: Marcel Dekker. 43-56.
51. Cohen J., J.R. Perfect, D.T. Durack. Cryptococcosis and the basidiospore. *Lancet.* ii:1301.
52. Coker, R. J. 1992. Cryptococcal infection in AIDS. *Int. J. STD AIDS* 3:168-172.
53. Collins H. L. and G. J. Bancroft 1991. Encapsulation of *Cryptococcus neoformans* impairs antigen-specific T cell responses. *Infect. Immun.* 59(11):3883-3888.
54. Constant S., D. Sant'Angelo, T. Pasqualini, T. Taylor, D. Levin, R. Flavell, and K. Bottomly. 1995. Peptide and protein antigens require distinct antigen presenting cell subsets fro the priming of CD4+ T cells. *J. Immunol.* 154:4915-4923.
55. Crabtree, G. R. 1989. Contingent genetic regulatory events in T lymphocyte activation. *Science* 243:355-360.
56. Cross C.E. and G.J. Bancroft. 1995. Ingestion of acapsular *Cryptococcus neoformans* occurs via mannose and B-glucan receptors, resulting in cytokine production and increased phagocytosis of the encapsulated form. *Infect. Immun.* 63:22604-2611.
57. Cross C.E., H.L. Collins, and G.J. Bancroft. 1997. CR3-dependent phagocytosis by murine macrophages: different cytokines regulate ingestion of a defined CR3 ligand and complement-opsonized *Cryptococcus neoformans*. *Immunol.* 91:289-296.

58. Crowe S.M., J.B. Carlin, K.R. Stewart., C.R. Lucas and J.F. Hoy. 1991. Predictive value of CD4 lymphocyte numbers for the development of opportunistic infections and malignancies in HIV-Infected persons. *J. AIDS.* 4:770-776.
59. Cumberbatch M., and I. Kimber. 1992. Dermal tumor necrosis factor- α induces dendritic cell migration to draining lymph nodes, and possibility provides one stimulus for Langerhans' cell migration. *Immunol.* 75:257-263.
60. Das K. C. and H.P. Misra, 1994., Impairment of raw 264.7 macrophage function by antiarrhythmic drugs. *Mol Cell Biochem* 132:151-162.
61. Davidson H.W. and C. Watts. 1989. Epitope-directed processing of specific antigen by B lymphocytes. *J. Cell. Biol.* 109:85-92.
62. Davidson H.W. and P.A.Reid. 1991. Processed antigen binds to newly synthesized MHC class II molecules in antigen-specific B lymphocytes. *Cell.* 67:105-116.
63. Davies S. R., DP. Clifford, J. R. Hoidal, and J.E. Repine. 1982. Opsonic requirements for the uptake of *Cryptococcus neoformans* by human polymorphonuclear leukocytes and monocytes. *J. Infect. Dis.* 145: 870-874.
64. de Waal Malefyt R., J. Abrams, B. Bennett, C.G. Figdor, and J.E. de Vries. 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocyte: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* 174:1209-1220.
65. del Prete G., M. De Carli, F. Almerigogna, M.G. Giudizi, R. Biagiotti, and S. Romagnani. 1993. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. 1993. *J. Immunol.* 150:353-360.
66. Demotz S. and A. Peleraux. 1996. Processing of DR1-restricted determinants from the fusion protein of measles virus following two distinct pathways. *Mol. Immunol.* 33:387-397.
67. Demotz S., P.M. Matricardi, C. Irle, P. Panina, A. Lanzavecchia, and G. Corradin. 1989. Processing of tetanus toxin by human antigen-presenting cells. *J. Immunol.* 143(12):3881-3886.
68. Denzin L.K. and P. Cresswell. 1995. HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. *Cell.* 82:155-165.
69. Deshaw M., and L.A. Pirofski. 1995. Antibodies to the *Cryptococcus neoformans* capsular glucuronoxylomannan are ubiquitous in serum from HIV+ and HIV- individuals. *Clin. Exp. Immunol.* 99(3):425-432.

70. Devi S.G.N., R. Schneerson, W. Egan, T.J. Ulrich, D. Bryla, J.B. Robbins, and J.E. Bennett. 1991. *Cryptococcus neoformans* serotype A glucuronoxylomannan-protein conjugate vaccines: synthesis, characterization and immunogenicity. *Infect. Immun.* 59:3700-3707.
71. Devi S.J. 1996. Preclinical efficacy of a glucuronoxylomannan-tetanus toxoid conjugate vaccine of *Cryptococcus neoformans* in a murine model. *Vaccine.* 14(9):841-844.
72. Diamond R.D. and J.E. Bennett, 1974. Prognostic factors in cryptococcal meningitis. A study in 111 cases. *Ann. Intern Med.* 80:176-181.
73. Diamond RD. 1989. *Cryptococcus neoformans*. In: Mandell GL, Douglas RC, Bennett J.E. (ed): Principles and practice of infectious diseases. John Wiley, New York. 1460-1468.
74. Diamond, R. D., and J. E. Bennett. 1973. Growth of *Cryptococcus neoformans* within human macrophages *in vitro*. *Infect. Immun.* 7:231-6.
75. Diment S. and P. Stahl. 1985. Macrophage endosomes contain proteases which degrade endocytosed protein ligands. *J. Biol. Chem.* 260:15311-15317.
76. Dismukes, W. E. 1988. Cryptococcal meningitis in patients with AIDS. *J. Infect. Dis.* 157:624-628.
77. Dodi A.I. S. Brett, T. Nordeng, S. Sidhu, R. Batchelor, G. Lombardi, O Bakke, and R.I. Lechler. 1994. The invariant chain inhibits presentation of endogenous antigens by a human fibroblast cell line. *Eur. J. Immunol.* 24:1632-1639.
78. Dong A.M. and J.W. Murphy 1996. Cryptococcal polysaccharides induce L-selectin shedding and tumor necrosis factor receptor loss from the surface of human neutrophils. *J. Clin Invest.* 97(2):689-698.
79. Dromer F., S. Mathoulin, B. Dupont, A. Laporte and the French Cryptococcosis study group. 1996a. Epidemiology of cryptococcosis in France: a 9-year survey (1985-1993). *Clin. Infect. Dis.* 23:82-90.
80. Dromer F., C. Perronne, J. Barge, J.L. Vilde and P. Yeni. 1989. Role of IgG and complement component C5 in the initial course of experimental cryptococcosis. *Clin. Exp. Immunol.* 78(3):412-417.
81. Dromer F., P. Aucouturier, J. Clauvel, G. Saimot, and P. Yeni. 1988. *Cryptococcus neoformans* antibody levels in patients with AIDS. *Scand J. Infect. Dis.* 20:283-285.
82. Dromer F., S. Mathoulin, B. Dupont, L. Letenneur., O. Ronin., and the French Cryptococcosis Study Group. 1996b. Individual and environmental factors associated

- with infection due to *Cryptococcus neoformans* serotype D. Clin. Infect. Dis. 23:91-96..
83. Dulic, V., L. F. Drullinger, E. Lees, S. T. Reed, and G. H. Stein. 1993. Altered regulation of G1 cyclins in senescent human diploid fibroblasts: accumulation of inactive cyclin E-cdk2 and cyclin D1-cdk2 complexes. Proc. Natl. Acad. Sci. USA 90:11034-11038.
 84. Dykstra, M. A., L. Friedman, and J. W. Murphy. 1977. Capsule size of *Cryptococcus neoformans*: control and relationship to virulence. Infect. Immun. 16:129-35.
 85. Egner W., Andreesen R., and D.N. Hart. Allostimulatory cells in fresh human blood: heterogeneity in antigen-presenting cell populations. 1993. Transplant. 56(4):945-950.
 86. Eissenberg LG, Goldman WE, Schlesinger PH. 1993. *Histoplasma capsulatum* modulates the acidification of phagolysosomes. J Exp Med. 177:1605-1611.
 87. Elliot W.L., S. Lu, Q. Nguyen, P.s. Reisert, T. Sairenji, C.H. Sorli, C.J. Stille, L.J. Thomas, and R.E. Humphreys. Hyperexpressed hairy leukemia cell li might bind to the antigen-presenting site of class II MHC molecules. Leukemia. 1:395-396.
 88. Ellis D.H. and T.J. Pfeiffer. 1990a. Natural habitat of *Cryptococcus neoformans* var. *gatti*. J. Clin. Microbiol. 28:1642-1644.
 89. Ellis D.H., and T. J. Pfeiffer. 1990b. Ecology, life cycle and infectious propagule of *Cryptococcus neoformans*. Lancet. 336:923-925.
 90. Eng, R. H., E. Bishburg, and S. M. Smith. 1986. Cryptococcal infection in patients with acquired immune deficiency syndrome. Am. J. Med. 81:19-23.
 91. Engering A.J., M. Cella, D.M. Fluitsma, E.C.M. Hoefsmit, A. Lanzavecchia, and J. Pieters. Mannose receptor mediated antigen uptake and presentation in human dendritic cells. 1997. Adv. Exp. Med. Biol. 417:183-187.
 92. Ezekowitz R.A., K. Sastry, P. Bailly, and A. Warner. 1990. Molecular characterization of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeasts in Cos-1 cells. J. Exp. Med. 172:1785-1794.
 93. Ezekowitz R.A.B., D.J. Williams, H. Koziel, M.Y.K. Armstrong. A. Warner, F.F. Richards, and R.M. Rose. 1991. Uptake of *Pneumocystis carinii* mediated by the macrophage mannose receptor. Nature. 351:155-158.
 94. Fahri F. G.S. Bulmer and T. R. Tacker 1970. *Cryptococcus neoformans*. IV. The not so encapsulated yeast. Infect. Immun. 1:526-531.

95. Fidel, P. L. and J. W. Murphy. 1990. Characterization of a cell population which amplifies the anticryptococcal delayed-type hypersensitivity response. *Infect. Immun.* 58:393-398.
96. Filgueira L. F.O. Nestle, M. Rittig, H.I. Joller, and P. Groscurth. 1996. Human dendritic cells phagocytose and process *Borrelia burgdorferi*. *J. Immunol.* 157:2998-3005.
97. Fineschi B., and J. Miller. 1997. Endosomal proteases and antigen processing. *TIBS.* 22(10):377-382.
98. Firpo, E. J., A. Koff, M. J. Solomon, and J. M. Roberts. 1994. Inactivation of Cdk2 inhibitor during interleukin 2-induced proliferation of human T lymphocytes. *Mol. and Cell. Biol.* 14:4889-4901.
99. Fleisher B, Schrenzenmeirer H, Conradt P. 1989. T lymphocyte activation by Staphylococcal enterotoxins: Role of class II molecules and T cell surface structures. *Cell Immunol.* 120:92-101.
100. Flesch, I.E.A., G. Schwamberger, and S.H.E. Kaufmann. 1989. Fungicidal activity of IFN- γ activated macrophages; extracellular killing of *Cryptococcus neoformans*. *J. Immunol.* 142:3219-3224.
101. Fremont D.L., M. Matsumura, E.A. Stura, and P.A. Wilson. 1992. Crystal structures of two viral peptides in complex with murine MHC class I H-2Kb. *Science.* 257:919-927.
102. Freudenthal P.S. and R.M. Steinman. 1990. The distinct surface of human blood dendritic cells, as observed after an improved isolation method. *Proc. Natl. Acad Sci USA.* 87:7698-7702.
103. Fromtling, R. A., H. J. Shadomy, and E. J. Jacobsen. 1982. Decreased virulence in stable, acapsular mutants of *Cryptococcus neoformans*. *Mycopathologia* 79:23-29.
104. Gajewski T.F. and F.W. Fitch. 1990. Anti-proliferative effect of IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper lymphocyte clones. *J. Immunol.* 140:4245-4252.
105. Gajewski T.F., M. Pinna, T. Wong, and F.W. Fitch. 1991. Murine Th1 and Th2 clones proliferate optimally in response to distinct antigen-presenting cell populations. *J. Immunol.* 146(6):1750-1758.
106. Galy A., M. Travis, D. Cen, and B. Chen. 1995. Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity.* 3:459-473.

107. Gapin L., Y. Bravo de Alba, A. Casrouge, J.P. Cabaniols, P. Kourilsky, and J. Kanellopoulos. 1998. Antigen presentation by dendritic cells focuses T cell response against immunodominant peptides: studies in the hen egg- white lysozyme (HEL) model. *J. Immunol.* 160:1555-1564.
108. Geppert TD, Lipsky PE. Regulatory role of microfilaments in the induction of T4 cell proliferation and interleukin 2 production. *Cell Immunol.* 1990; 131:205-218.
109. Germain R.N. 1981. Accessory cell stimulation of T cell proliferation requires active antigen processing, Ia-restricted antigen presentation, and a separate nonspecific second signal. *J. Immunol.* 127(5):1964-1966.
110. Germain R.N. 1994. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell.* 76:287-299.
111. Germain, R. N. 1986. The ins and outs of antigen processing and presentation. *Nature* 322:687-689.
112. Gonzalez-Fernandez M., E. Carrasco-Marin, C. Alvarez-Dominguez, I.M. Outschoorn, and F. Leyva-Cobian. 1997. Inhibitory effects of thymus-independent type 2 antigens on MHC class II-restricted antigen presentation: comparative analysis of carbohydrate structures and the antigen presenting cell. *Cell. Immunol.* 171:1-113.
113. Gordon A.H., P. D'Arcy Hart, and M.R. Young. 1980. Ammonia inhibits phagosome-lysosome fusion in macrophages. *Nature.* 286:79-80.
114. Granger, D. L., J. R. Perfect, and D. T. Durack. 1985. Virulence of *Cryptococcus neoformans*: regulation of capsule synthesis by carbon dioxide. *J. Clin. Invest.* 76:508-16.
115. Graybill J.R. and L. Mitchell. 1979. Host defense in Cryptococcosis. III. *In vivo* alteration of immunity. *Mycopath.* 69(3):171-178.
116. Graybill, J. R. and R. H. Alford. 1974. Cell mediated immunity in cryptococcosis. *Cell. Immunol.* 14:12-21.
117. Graybill, J.R. and D.J. Drutz. 1978. Host defense in cryptococcosis. II. Cryptococcosis in the nude mouse. *Cell. Immunol.* 40:263-274.
118. Green G.M. and E.H. Kass. 1963. The role of the alveolar macrophage in the clearance of bacteria from the lung. *J. Exp. Med.* 119:167-173.
119. Groux, H., G. Torpier, D. Monte, Y. Mouton, A. Capron, and J. C. Ameisen. 1992. Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-induced asymptomatic individuals. *J. Exp. Med.* 175:331-334.

120. Gruenberg J. and F.R. Maxfield. 1995. Membrane transport in the endocytic pathway. *Curr. Opin. Immunol.* 7:552-563.
121. Guagliardi L.E., B. Koppelman, J.S. Blum, M.S. Marks, P. Cresswell, and F.M. Brodsky. 1990. Co-localization of molecules involved in antigen processing and presentation in an early endocytic compartment. *Nature.* 343:133-139.
122. Guzman C.A., M. Rohde, M. Bock and K.N. Timmis. 1994. Invasion and intracellular survival of *Bordetella bronchiseptica* in mouse dendritic cells. *Infect. Immun.* 62(12):5528-5537.
123. Guzman C.A., M. Rohde, T. Chakraborty, E. Domann, M. Hudel, J. Wehland and K. N. Timmis. 1995. Interaction of *Listeria monocytogenes* with mouse dendritic cells. *Infect. Immun.* 63(9):3665-3673.
124. Harding C.V. 1993. Cellular and molecular aspects of antigen processing and the function of class II MHC molecules. *Am. J. Respir. Cell. Mol. Biol.* 8:461-467.
125. Harmsen A.G., B.H. Muggenburg, M. Burton Snipes, D.E. Bice. 1985 The role of macrophages in particle translocation from lungs to lymph nodes. *Science.* 320:21277-1280.
126. Havenith C.E., J.J. van Haarst, A.J. Breedijk, M.G. Betjes, H.C. Hoogsteden, R.H. Beelen, and E.C. Hoefsmit. 1994. Enrichment and characterization of dendritic cells from human bronchoalveolar lavages. *Clin. Exp. Immunol.* 86:339-343.
127. Havenith C.E.G., A.J. Breedijk, M.G.H. Betjes, W. Calame, R.H.J. Beelen, and E.C.M. Hoefsmit. 1993a. T cell priming in situ by intratracheally instilled antigen-pulsed dendritic cell. *Am. J. Respir. Cell Mol. Biol.* 8:319-324.
128. Havenith C.E.G., P.P.M.C. van Miert, A.J. Breedijk, R.H.J. Beelen, and E.C.M. Hoefsmit. 1993b. Migration of dendritic cells into the draining lymph nodes of the lung after intratracheal instillation. *Am J. Respir. Cell Mol. Biol.* 9:484-488.
129. Henderson R.A., S.C. Watkins, and J.L. Flynn. 1997. Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*. *J. Immunol.* 159:635-643.
130. Herrmann J.L., N. Dubois, M. Fourgeaud, D. Basset, and P.H. Lagrange. 1994. Synergic inhibitory activity of amphotericin-B and gamma interferon against intracellular *Cryptococcus neoformans* in murine macrophages. *J. Antimicro. Chemother.* 34(6):1051-1058.
131. Hill J.O. and P.L. Dunn. 1993. A T cell-independent protective host response against *Cryptococcus neoformans* expressed at the primary site of infection in the lung. *Infect. Immun.* 61(12):5302-5308.

132. Hill, J. O. 1992. CD4+ T cells cause multinucleated giant cells to form around *Cryptococcus neoformans* and confine the yeast within the primary site of infection in the respiratory tract. *J. Exp. Med.* 175:1685-1695.
133. Hill, J. O. and A. G. Harmsen. 1991. Intrapulmonary growth and dissemination of an avirulent strain of *Cryptococcus neoformans* in mice depleted of CD4+ or CD8+ T cells. *J. Exp. Med.* 173:755-758.
134. Holt P.G. and M.A. Schon-Hegrad. 1987. Localization of T cells, macrophages, and dendritic cells in rat respiratory tract tissue: implications for immune function studies. *Immunol.* 62:349-256..
135. Houpt D.G. and G.S. Pfrommer, B.J. Young, T.A. Larson and T.R. Kozel. 1994. Occurrences immunoglobulin classes and biological activities of antibodies in normal human serum that are reactive with *Cryptococcus neoformans* glucuroxylomannan. *Infect and Immun.* 62(7):2857-2864.
136. Hoy, J. F., D. E. Lewis, and G.P.G. Miller. 1988. Functional versus phenotypic analysis of T cells in subjects seropositive for the human immunodeficiency virus: A prospective study of *in vitro* responses to *C. neoformans*. *J. Infect. Dis.* 158:1071-1078.
137. Hoy, J. F., J. W. Murphy, and G.G. Miller. 1989. T cell response to soluble cryptococcal antigens after recovery from cryptococcal infection. *J. Infect. Dis.* 159:116-9.
138. Huffnagle G.B., G.B. Toews, M.D. Burdick, M.B. Boyd, K.S. McAllister, R.A. McDonald, S.L. Kunkel, and R.M. Strieter. 1996. Afferent phase production of TNF- α is required for the development of protective T cell immunity to *Cryptococcus neoformans*. *J. Immunol.* 157:4529-4536.
139. Huffnagle G.B., J.L. Yates, and M.F. Lipscomb. 1991a. T cell-mediated immunity in the lung: a *Cryptococcus neoformans* pulmonary infection model using SCID and athymic nude mice. *Infect. Immun.* 59(4):1423-1433.
140. Huffnagle G.B., M.B. Boyd, N.E. Street, and M.F. Lipscomb. 1998. IL-5 is required for eosinophil recruitment, crystal deposition, and mononuclear cell recruitment during a pulmonary *Cryptococcus neoformans* infection in genetically susceptible mice (C57BL/6). *J. Immunol.* 160:2393-2400.
141. Huffnagle G.B., R.M. Strieter, L.K. McNeil, R.A. McDonald, M.D. Burdick, S.L. Kunkel, and G.B. Toews. 1997. Macrophage inflammatory protein-1alpha (MIP-1alpha) is required for the efferent phase of pulmonary cell-mediated immunity to a *Cryptococcus neoformans* infection. *J. Immunol.* 159:318-327.

142. Huffnagle, G. B., J. L. Yates, and M. F. Lipscomb. 1991b. Immunity to a pulmonary *Cryptococcus neoformans* infection requires both CD4+ and CD8+ T cells. *J. Exp. Med.* 173:793-800.
143. Huffnagle, G. B., M. F. Lipscomb, J. A. Lovchik, K. A. Hoag, and N. E. Street. 1994. The role of CD4+ and CD8+ T cells in the protective inflammatory response to a pulmonary cryptococcal infection. *J. Leuk. Biol.* 55:35-42.
144. Imwidthaya P. 1994. One year's experience with *Cryptococcus neoformans* in Thailand. *Trans. R. Soc. Trop. Med. Hyg.* 88:208.
145. Inaba K., M. Inaba, M. Naito, and R.M. Steinman. 1993. Dendritic cell progenitors phagocytosis particulates including bacillus calmette-guerin organism and sensitize mice to mycobacterial antigens *in vivo*. *J. Exp. Med.* 178:479-488.
146. Iseki, R., M. Mukai, and M. Iwata. 1991. Regulation of T lymphocyte apoptosis. Signals for the antagonism between activation- and glucocorticoid-induced death. *J. Immunol.* 147:4286-4292.
147. Jackman M., M. Firth, and J. Pines. 1995. Human cyclins B1 and B2 are localized to strikingly different structures: B1 to microtubules, B2 primarily to the golgi apparatus. *EMBO J.* 14:1646-1654.
148. Jacobson, E. S., D. J. Ayers, A. C. Harrell, and C. C. Nicholas. 1982. Genetic and phenotypic characterization of capsule mutants of *Cryptococcus neoformans*. *J. Bacteriol.* 150:1292-1296.
149. Jacquier-Sarlin M.R., F.M. Gabert, M.B. Villers, and M.G. Colomb. 1995. Modulation of antigen processing and presentation by covalently linked complement C3b fragment. *Immunol.* 84:164-170.
150. Jaraquemada, D., M. Marti, and E.O. Long. 1990. An endogenous processing pathway in vaccinia virus infected cells for presentation of cytoplasmic antigens to class II restricted T cells. *J. Exp. Med.* 172:947-954.
151. Katula K.S., K.L. Wright, H. Paul, D.R. Surman, F.J. Nuckolls, J.W. Smith, J.P. Ting, J. Yates and J.P. Cogswell. 1997. Cyclin-dependent kinase activation and S-phase induction of the cyclin B1 gene are linked through the CCAAT elements. *Cell Growth Diff.* 8:811-820.
152. Kawakami K., S. Kohno, J. Kadota, M. Tohyama, K. Teruya, N. Kudenken, A. Saito, and K. Hara. 1995. T cell-dependent activation of macrophages and enhancement of their phagocytic activity in the lungs of mice inoculated with heat-killed *Cryptococcus neoformans*: involvement of IFN-gamma and its protective effect against cryptococcal infection. *Microbiol. Immunol.* 39:135-143.

153. Kaye P.M., B.M. Chain, and M. Feldmann. 1985. Nonphagocytic dendritic cells are effective accessory cells for anti-mycobacterial responses *in vitro*. *J. Immunol.* 134:1930-1934.
154. Kirschke H., A.J. Barrett, and N.D. Rawlings. 1995. Proteinases 1: lysosomal cysteine proteinases. *Protein Profile.* 2:1581-1643.
155. Kleijmeer M.J., M.A. Ossevoort, C.J. Van-Veen, J.J. Van-Hellemond, J.J. Neefjes, W.M. Kast, C.J. Melief, and H.J. Geuze. 1995. MHC class II compartments and the kinetics of antigen presentation in activated mouse spleen dendritic cells. *J. Immunol.* 154:5715-5724.
156. Knight F.R., D.W. Mackenzie, B.G. Evans, K. Porter, N.J. Barrett, G.C. White. Increasing incidence of cryptococcosis in the United Kingdom. 1993. *J. Infect.* 27:185-191.
157. Knight S.C., A. Stagg, S. Hill, P. Fryer, and S. Griffiths. 1992. Development and function of dendritic cells in health and disease. *J. Invest. Dermatol.* 99:33S-38S.
158. Koff, A., F. Cross, A. Fisher, J. Sehumacher, K. Leguellec, M. Phillippe, and J. M. Roberts. 1991. Human cyclin E, a new cyclin that interacts with two members of the *cdc2* gene family. *Cell* 66:1217-1228.
159. Koff, A., M. Ohtsuki, K. Polyak, J. M. Roberts, and J. Massague. 1993. Negative regulation of G1 progression in mammalian cells: inhibition of cyclin E-dependent kinase by TGF- β . *Science* 260:536.
160. Kovacs, J. A., A. A. Kovacs, M. Polis, W. C. Wright, V. J. Gill, C. U. Tuazon, E. P. Gelman, H. C. Lane, R. Longfield, E. G. Overturf, A. M. Macher, A. S. Fauci, J. E. Parrillo, J. E. Bennett, and H. Masur. 1985. Cryptococcosis in the acquired immune deficiency syndrome. *Ann. Int. Med.* 103:533-538.
161. Kovacsovics-Bankowski and K.L. Rock. 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science.* 267:243-245.
162. Kozel T. R., G.S.T. Pfrommer, A.S. Guerlain, B.A. Highison, and G.J. Highison. 1988b. Strain variation in phagocytosis of *Cryptococcus neoformans*: dissociation of susceptibility to phagocytosis from activation and binding of opsonic fragments of C3. *Infect. Immun.* 56:2794-2800.
163. Kozel T. R., G.S.T. Pfrommer, A.S. Guerlain, B.A. Highison, and G.J. Highison. 1988a. Role of the capsule in phagocytosis of *Cryptococcus neoformans*. *Rev. Infect. Dis* 10(Suppl):436-439.
164. Kozel T.R. and C.A. Harmerath. 1984. Binding of cryptococcal polysaccharide to *Cryptococcus neoformans*. *Infect. Immun.* 43:879-886.

- 165.Kozel T.R. and R. P. Mastroianni, 1976. Inhibition of phagocytosis by cryptococcal polysaccharide: dissociation of the attachment and ingestion phases of phagocytosis. *Infect. Immun.* 14: 62-67.
- 166.Kozel T.R. and T.G. McGaw, 1979. Opsonization of *Cryptococcus neoformans* by human immunoglobulin G.: role of immunoglobulin G in phagocytosis by macrophages. *Infect. Immun.* 25:255-261.
- 167.Kozel T.R. W.F. Gulley, and J. Cazin. 1977. Immune response to *Cryptococcus neoformans* soluble polysaccharide: immunological unresponsiveness. *Infect Immun.* 18:701-707.
- 168.Kozel TR. 1977. Non-encapsulated variant of *Cryptococcus neoformans*. II. Surface receptors for cryptococcal polysaccharide and their role in inhibition of phagocytosis by polysaccharide. *Infect. Immun.* 16:99-106.
- 169.Kozel, T. R., and J. Cazin. 1971. Nonencapsulated variant of *Cryptococcus neoformans*. I. Virulence studies of characterization of soluble polysaccharide. *Infect. Immun.* 3:287-294.
- 170.Kozel, T. R., B. Highison, and J. C. Stratton. 1984. Localization on encapsulated *Cryptococcus neoformans* of serum components opsonic for phagocytosis by macrophages and neutrophils. *Infect. Immun.* 43:574-9.
- 171.Kreiger J.I., S.F. Grammer, H.M. Gray and RAW. Chestnut. 1985. Antigen presentation by splenic B cells: resting B cells are ineffective whereas activated B cells are effective accessory cells for T cell responses. *J. Immunol.* 135:2937-2946.
- 172.Kuchroo, V.K., M.P. Das, J.A. Brown, A.M. Ranger, S.S. Zamvil, R.A. Sobel, H.L. Weiner, N. Nabavi, and L.H. Glimcher. 1995. B71 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways. *Cell.* 80: 707-718.
- 173.Kumagai, N., S. H. Benedict, G. B. Mills, and E. W. Gelfand. 1987. Requirements for the simultaneous presence of phorbol esters and calcium ionophores in the expression of human T lymphocyte proliferation-related genes. *J. Immunol.* 139:1393-1399.
- 174.Kwon-Chung K.J. 1980. Nuclear genotypes of spore chains in *Filobasidiella neoformans* (*Cryptococcus neoformans*). *Mycologia.* 72:418-422.
- 175.Lambrecht B.N., R.A. Pauwels, and G.R. Bullock. 1996. The dendritic cell: its potent role in the respiratory immune response. *Cell Biol. Int.* 20(2):111-120.
- 176.Lanzavecchia A. 1990. Receptor-mediated antigen uptake and its effect on antigen presentation to class II restricted T lymphocytes. *Ann Rev Immunol.* 8:733-793.

- 177.Lanzavecchia A. 1996. Mechanisms of antigen uptake for presentation. *Curr. Opin. Immunol.* 8:348-354.
- 178.Larsen C.P., P.J. Morris, and J.M. Austyn. 1990. Donor dendritic leukocytes migrate from cardiac allograft into recipients' spleens. *Transplant. Proc.* 22:1943-1944.
- 179.Larsen C.P., S.C. Ritchie, T.C. Pearson, P.S. Linsley, and R.P. Lowry. 1992. Functional expression of the costimulatory molecule, B7/BB1, on murine dendritic cell populations. *J. Exp. Med.* 176:1215-1220.
- 180.Larsson M., J. Berge, A.G. Johansson, U. Forsum. 1997. Human dendritic cells handling of binding, uptake and degradation of free and IgG-immune complexed dinitrophenylated human serum albumin *in vitro*. *Immunol.* 90:138-146.
- 181.Lasalle J.M. and D.A. Hafler. 1994. T cell Anergy. *FASEB J.* 8:601-608.
- 182.Laxalt K.A. and T.R. Kozel. 1979. Chemotaxigenic and activation of the alternative complement pathway by encapsulated and nonencapsulated *Cryptococcus neoformans*. *Infect. Immun.* 26:435-440.
- 183.Lazcano O., V.O. Speights, J.G. Strickler, J.E. Bilbao, J. Becker and J. Diaz. 1993. Combined histochemical stains in the differential diagnosis of *Cryptococcus neoformans*. *Mod. Pathol.* 6(1):80-84.
- 184.Lee S.C., Kress Y., Zhao M.L., Dickson D.W., and Casadevall A. 1995 *Cryptococcus neoformans* survive and replicate in human microglia. *Lab Invest* 73, 871-879.
- 185.Lee, J. C., A. Trueneh, M. F. Smith, and K. Y. Tsang. 1987. Induction of interleukin 2 receptor (Tac) by tumor necrosis factor in YT cells. *J. Immunol.* 139:1935-1938.
- 186.Legaard P.K., R.D. Legrand, and M.L. Misfeldt. 1991. The superantigen *Pseudomonas* exotoxin A requires additional functions from accessory cells for T lymphocyte proliferation. *Cell Immunol.* 1991;135:372-382.
- 187.Legaard P.K., R.D. Legrand, and M.L. Misfeldt. 1992. Lymphoproliferative activity of *Pseudomonas* Exotoxin A is dependent on intracellular processing and is associated with the carbonyl-terminal portion. *Infect. Immun.* 60(4):1273-1278.
- 188.Lenardo, M. J. 1991. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature* 353:858-861.
- 189.Lenshow D.J., A.I. Sperling, M.P. Cooke, G. Freeman, L. Rhee, D.C. Decker, G. Gray, L.M. Nadler, C.C. Goodnow, and J.A. Bluestone. 1995. Differential up-regulation of the B7-1 and B7-2 costimulatory molecules after Ig receptor engagement by antigen. *J. Immunol.* 153:1990-1997.

190. Levine T.P. and B.M. Chain. 1992. Endocytosis by antigen presenting cells: dendritic cells are as endocytically active as other antigen presenting cells. Proc. Natl. Acad. Sci. USA. 89:8343-8346.
191. Levitz S.M. and A. Tabuni, 1991. Binding of *Cryptococcus neoformans* by human cultured macrophages. Requirements for multiple complement receptors and actin. J. Clin Invest. 87(2):258-535.
192. Levitz S.M. and D.J. Dibeneditto, 1988. Differential stimulation of murine resident peritoneal cells by selectively opsonized encapsulated and acapsular *Cryptococcus neoformans*. Infect Immun. 56:2544-2551.
193. Levitz S.M. 1991a. The Ecology of *Cryptococcus neoformans* and the Epidemiology of Cryptococcosis. Rev. Infect. Dis. 13:1163-1169.
194. Levitz S.M. 1991b. Activation of human peripheral blood mononuclear cells by interleukin 2 and granulocyte-monocyte colony stimulating factor to inhibit *Cryptococcus neoformans*. Infect Immun. 59, 3393-3397.
195. Levitz S.M. and T.P. Farrell. 1990. Growth inhibition of *Cryptococcus neoformans* by cultured human monocytes: role of the capsule opsonins, the culture surface and cytokines. Infect. Immun. 58:1201-1209.
196. Levitz S.M., A. Tabuni, and C. Treseler. 1993. Effect of mannose-binding protein on binding of *Cryptococcus neoformans* to phagocytes. Infect. Immun. 61:4891-4893.
197. Levitz, S. M. and M. P. Dupont. 1993. Phenotypic and functional characterization of human lymphocytes activated by interleukin-2 to directly inhibit growth of *Cryptococcus neoformans in vitro*. J. Clin. Invest. 91: 1490-1498.
198. Levitz, S. M., A. Tabuni, H. Kornfeld, C. C. Reardon, and D. T. Golenbock. 1994a. Production of tumor necrosis factor alpha in human leukocytes stimulated by *Cryptococcus neoformans*. Infect. Immun. 62:1975-1981.
199. Levitz, S. M., M. P. Dupont, and E. H. Small. 1994b. Direct activity of human T lymphocytes and natural killer cells against *Cryptococcus neoformans*. Infect. Immun. 62:194-202.
200. Lewis, J.L. and S. Rabinovich. 1972. The Wide Spectrum of Cryptococcal Infections. Amer. J. Med. 53:315-322.
201. Lim T.S. and J.W. Murphy. 1980. Transfer of immunity to cryptococcosis by T-enriched splenic lymphocytes from *Cryptococcus neoformans*-sensitized mice. Infect. Immun. 30:5-11.

202. Lipscomb M.F. 1989. Lung defenses against opportunistic infections. *Chest*. 96:1393-1399.
203. Liu Y.B. and C.A. Janeway. 1991. Microbial induction of costimulatory activity for CD4 T cell growth. *Int. Immunol.* 3:323-332.
204. Lopes, M. F., V. F. da Veuga, A. R. Santos, N. E. Fonseca, and G. A. Dos Reis. 1995. Activation induced CD4+ T cell death by apoptosis in experimental Chagas' disease. *J. Immunol.* 154:744-752.
205. Lorenz R.G., J.S. Blum, and P.M. Allen. 1990. Constitutive competition by self proteins for antigen presentation can be overcome by receptor-enhanced uptake. *J. Immunol.* 144:1600-1606.
206. Lotteau V. L. Teyton, A. Peleraux, T. Nilsson, L. Karlsson, S.L. Schmid, V. Quarant, and P.A. Peterson. 1990. Intracellular transport of class II MHC molecules directed by invariant chain. *Nature.* 348:600-605.
207. Lutz M.B., P. Rovere, M.J. Kleijmeer, M. Rescigno, C.U. Abmann, V.M.J. Oorschot, H.J. Geuze, J. Trucy, D. Demandolx, J. Davoust, and P. Ricciardi-Castagnoli. 1997. Intracellular routes and selective retention of antigens in mildly acidic cathepsin D/lysosome-associated membrane protein-1/MHC class II-positive vesicles in immature dendritic cells. *J. Immunol.* 159:3707-3716.
208. Lyman C.A. and T.J. Walsh. 1992. Systemically administered antifungal agents. A review of their clinical pharmacology and therapeutic applications. *Drugs.* 44:9-35. flucytosine resistance
209. Manca F., D. Fenglio, G. Li Pira, A. Kunkl, and F. Celada. 1991. Effect of antigen/antibody ratio on macrophage uptake, processing, and presentation to T cells of antigen complexed with polyclonal antibodies. *J. Exp. Med.* 173:37-48.
210. Maraskovsky E., K. Brasel, M. Teepe, E.R. Roux, S.D. Lyman, K. Shortman and H.J. McKenna. 1996. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J. Exp. Med.* 184: 1953-1962.
211. Maric M.A., M. D. Taylor and J. S. Blum. 1994. Endosomal aspartic proteinases are required for invariant-chain processing. *PNAS.* 91:2171-2175.
212. Matsushime, H., M. Roussel, R. Ashmun, and C. J. Sherr. 1991. Colony stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* 65:701-713.

213. Mazingue, C., C. Walker, W. Domzig, A. Capron, A. De Weck, and B. M. Stadler. 1987. Effect of schistosome-derived inhibitory factor on the cell cycle of T lymphocytes. *Int. Arch. Allergy Appl. Immunol.* 83:12-18.
214. Mbow M.L., N. Zeidner, N. Panella, R.G. Titus, and J. Piesman. 1997. *Borrelia burgdoferi*-pulsed dendritic cells induce a protective immune response against tick-transmitted spirochetes. *Infect. Immun.* 65(8):3386-3390.
215. McCombs C.C., J.P. Michalski, B.T. Westerfield, R.A.W. Light. 1982. Human alveolar macrophages suppress the proliferative response of peripheral blood lymphocytes. *Chest.* 82(3):266-271.
216. McCoy K.L., M.S. Page, B.J. Merkel, J.K. Inman, and R. Stutzman. 1993. Differences among various lineages of antigen-presenting cells in processing exogenous antigen internalized through transferrin receptors. *J. Immunol.* 151:6757-6768.
217. McWilliams A.S., D. Nelson, J.A. Thomas and P.G. Holt. 1994. Rapid dendritic cell recruitment is a hallmark of the nature inflammatory response at mucosal surfaces. *J. Exp. Med.* 179:1331-1336.
218. Metalay J. P., E. Pure, and R. M. Steinman. 1989. Control of the immune response at the level of antigen-presenting cells: a comparison of the function of dendritic cells and B cells. *Adv. Immunol.* 47:45-111.
219. Miller, G.P.G. and J. Puck. 1984. *In vitro* human lymphocyte responses to *Cryptococcus neoformans*. Evidence for primary and secondary responses in normals and infected subjects. *J. Immunol.* 133:166-172.
220. Mitchell T.G. and J.R. Perfect. 1995. Cryptococcosis in the era of AIDS - 100 years after the discovery of *Cryptococcus neoformans*. *Clin. Micro. Rev.* 8:515-548.
221. Mitchell, T. G., and L. Friedman. 1972. *In vitro* phagocytosis and intracellular fate of variously encapsulated strains of *Cryptococcus neoformans*. *Infect. Immun.* 5:491-8.
222. Modiano, J. F., J. Domenico, A. Szepesi, J. J. Lucas, and E. W. Gelfand. 1994. Differential requirements for interleukin-2 distinguish the expression of an activity of the cyclin dependent kinases Cdk4 and Cdk2 in human T cells. *J. Biol. Chem.* 269:32972-32978.
223. Mody C.H., G.B. Toews. and M.F. Lipscomb. 1988. Treatment of murine cryptococcosis with cyclosporin-A in normal and athymic mice. *Am. Rev. Respir. Dis.* 139:7-12.
224. Mody C.H., K.L. Sims, C.J. Wood, R.M. Syme, J.C.L. Spurrell, and M.M. Sexton. 1996. Proteins in the cell wall and membrane of *Cryptococcus neoformans* stimulate

- lymphocytes from both adults and fetal cord blood to proliferate. *Infect. Immun.* 64(11):4811-4819.
225. Mody, C. H. and R. M. Syme. 1993. Effect of polysaccharide capsule and methods of preparation on human lymphocyte proliferation in response to *Cryptococcus neoformans*. *Infect. Immun.* 61:464-469.
226. Mody, C. H., C. L. Tyler, R. G. Sitrin, C. Jackson, and G. B. Toews. 1991. Interferon-gamma activates rat alveolar macrophages for anticryptococcal activity. *Am. J. Resp. Cell Mol. Biol.* 5:19-26.
227. Mody, C. H., G. H. Chen, C. J. Jackson, J. L. Curtis, and G. B. Toews. 1993. Depletion of murine CD8+ T cells *in vivo* decreases pulmonary clearance of a moderately virulent strain of *Cryptococcus neoformans*. *J. Lab. Clin. Med.* 121:765-774.
228. Mody, C. H., M. F. Lipscomb, and G. B. Toews. 1990. Depletion of CD4+ (L3T4+) lymphocytes *in vivo* impairs murine host defense to *Cryptococcus neoformans*. *J. Immunol.* 144:1472-1477.
229. Mody, C. H., R. Paine, C. J. Jackson, G. H. Chen and G. B. Toews. 1994. CD8 cells play a critical role in delayed type hypersensitivity to intact *Cryptococcus neoformans*. *J. Immunol.* 152:3970-3977.
230. Moll H., S. Flohe, and M. Rølinghoff. 1995. Dendritic cells in *Leishmania major* infected mice harbor persistent parasites and mediate an antigen-specific T cell immune response. *E. J. Immunol.* 25:693-699.
231. Monaco JJ. 1995. Pathways for the processing and presentation of antigens to T cells. *J. Leuk. Biol.* 57:543-547.
232. Monari C., C. Retini, B. Palazzetti, F. Bistoni, and A. Vecchiarelli. 1997. Regulatory role of exogenous IL-10 in the development of immune response versus *Cryptococcus neoformans*. *Clin. Exp. Immunol.* 109:242-247.
233. Monga D.P. 1981. Role of macrophages in resistance of mice to experimental cryptococcosis. *Infect. Immun.* 32:975-978.
234. Mootsikapun P., P. Chetchotisakd, and B. Intarapoka. 1996. Pulmonary infections in HIV infected patients. *J. Med. Assoc. Thai.* 79(8):477-485.
235. Morel A., S. Quaratino, D.C. Douek and M. Londei. 1997. Split activity of interleukin-10 on antigen capture and antigen presentation by human dendritic cells: definition of a maturative step. *Eur. J. Immunol.* 27:26-34.

236. Moreno J. and Lipsky PE. Differential ability of fixed antigen-presenting cells to stimulate nominal antigen-reactive and alloreactive T4 lymphocytes. *J. Immunol.* 1986;136:3579-3587.
237. Morrison, L. A., A. E. Lukacher, V. L. Braciale, D. P. Fan, and T. J. Braciale. 1986. Differences in antigen presentation to MHC class I and class II restricted influenzae virus specific cytolytic T clones. *J. Exp. Med.* 163:903-921.
238. Mosmann T.R., and R.L. Coffman. 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Ann. Rev. Immunol.* 7:145-173.
239. Mozaffarian N. J.W. Berman and A. Casadevall. 1995. Immune complexes increase nitric oxide production by interferon-gamma stimulated murine macrophage-like J774.16 cells. *J. Leuk. Biol.* 57:657-662.
240. Mukherjee J., G. Nussbaum, M.D. Scharff, and A. Casadevall. 1995. Protective and nonprotective monoclonal antibodies to *Cryptococcus neoformans* originating from one B cell. *J. Exp. Med.* 181:405-409.
241. Mukherjee S., S. Lee, J. Mukherjee, M.D. Scharff, and A. Casadevall. 1994. Monoclonal antibodies to *Cryptococcus neoformans* capsular polysaccharide modify the course of intravenous infection in mice. *Infect. Immun.* 62(3):1079-1088.
242. Murphy J.W. 1988. Influence of cryptococcal antigens on cell-mediated immunity. *Rev. Inf Dis.* 10:S432-435.
243. Murphy J.W. 1993. Cytokine profiles associated with induction of anticryptococcal cell mediated immune response. *Infect. Immun.* 61:4750-4759.
244. Murphy J.W., A. Zhou, and S.C. Wong. 1997. Direct interactions of human natural killer cells with *Cryptococcus neoformans* inhibit granulocyte-macrophage colony-stimulating factor and tumor necrosis factor alpha production. *Infect. Immun.* 65:4564-4571.
245. Murphy J.W., and J.W. Moorhead. 1982. Regulation of cell-mediated immunity in cryptococcosis. I. Induction of specific afferent T suppressor cells by cryptococcal antigen. *J. Immunol.* 128:276-282.
246. Murphy J.W., and R.L. Mosley. 1985. Regulation of cell-mediated immunity in cryptococcosis. III. Characterization of Second-Order T suppressor cells (Ts2). *J. Immunol.* 134(1):577-584.
247. Murphy, J.W. and D.O. McDaniel. 1982. *In vitro* reactivity of natural killer (NK) cells against *Cryptococcus neoformans*. *J. Immunol.* 128: 1577-1583.

248. Murphy, J.W., and G.C. Cozad. 1972. Immunological unresponsiveness induced by cryptococcal polysaccharide assayed by the hemolytic plaque technique. *Infect. Immun.* 5:896-901.
249. Murray J.F., and J. Mills. 1990. Pulmonary infectious complications of human immunodeficiency virus infection. *Am Rev Respir Dis*; 141:1582-1598.
250. Nair S., A.M. Buiting, R.J. Rouse, N. Van Rooijen, L. Huang, and B.T. Rouse. 1995. Role of macrophages and dendritic cells in primary cytotoxic T lymphocyte responses. *Int. Immunol.* 7:679-688.
251. Neckers, L. M. and J. Cossman. 1983. Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2. *Proc. Natl. Acad. Sci. USA.* 80:3494-3498.
252. Neefjes J.J. and H. Ploegh. 1992. Inhibition of endosomal proteolytic activity by leupeptin blocks surface expression of MHC class II molecules and their conversion to SDS resistant $\alpha\beta$ heterodimers in endosomes. *EMBO J.* 11:411-416.
253. Newman W., S.R. Targan, and L.D. Fast. 1984. Immunobiological and immunochemical aspects of the T-200 family of glycoproteins. *Mol. Immunol.* 21:1113-1121.
254. Nicod L.P., M.F. Lipscomb, J.C. Weissler, C.R. Lyons, J. Albertson, and G. Toews. 1987. Mononuclear cells in human lung parenchyma. *Am. Rev. Respir. Dis.* 136:818-823.
255. Nijman H.W., M.J. Kleijmeer, M. A. Ossevoort, V.M.J. Oorschot, M.P.M. Vierboom, J. van de Keur, P. Kenemans, W. Martin Kast, H.J. Geuze, and C. J.M. Melief. 1995. Antigen capture and major histocompatibility class II compartments of freshly isolated and cultured human blood dendritic cells. *J. Exp. Med.* 182:163-174.
256. Norbury C.C., L.J. Hewlett, AR. Prescott, N. Shastri, and C. Watts. 1995. Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity.* 3:783-791.
257. O'Doherty U, Steinman R.M., Peng M, Cameron P.U., Gezelter S, Kopeloff I, Swiggard W.J., Pope M, Bhardwaj N. 1993. Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. *J Exp Med.* 178:1067-1078.
258. Ohtsubo, M. and J. Roberts. 1993. Cyclin dependent regulation of G1 in mammalian fibroblasts. *Science* 259:1908-1912.

259. Ossevoort M.A., R.E.M. Toes, M.L.H. De Bruijn, C.J.M. Melief, C.G. Figdor, and W.M. Kast. 1992. A rapid isolation procedure for dendritic cells from mouse spleen by centrifugal elutriation. *J. Immunol. Meth.* 155: 101-111.
260. Paine, R. I., C. H. Mody, A. Chavis, M. A. Spahr, L. A. Turka, and G. B. Toews. 1991. Alveolar epithelial cells block lymphocyte proliferation *in vitro* without inhibiting activation. *Am. J. Resp. Cell Mol. Biol.* 5:221-229.
261. Pancake S.J. and S.G. Nathenson. 1973. Selective loss of H-2 antigenic reactivity after chemical modification.
262. Paul W.E. and R.A. Seder. 1994. Lymphocyte responses and cytokines. *Cell.* 76:241-251.
263. Pechold K. and D. Kabelitz. 1993. Human peripheral blood gamma delta T cells are uniformly sensitive to destruction by the lysosomotropic agents leucine methyl ester and leucyl leucine methyl ester. *Eur. J. Immunol.* 23(2):562-565.
264. Peetermans W., H. Bobbaers, J. Verhaegen, and J. Vadepitte. 1993. fluconazole-resistant *Cryptococcus neoformans var. gatti* in an AIDS patient. *Acta. Clinica. Belgica.* 48:405-409.
265. Perfect J.R. 1989. Cryptococcosis. *Infect. Clin. N. Am.* 3:77-102.
266. Perfect, J. R., D. L. Granger, and D. T. Durack. 1987. Effects of antifungal agents and g-interferon on macrophage cytotoxicity for fungi and tumor cells. *J. Infect. Dis.* 156:316-323.
267. Pfeifer JD, Wick MF, Roberts R.L., Findlay K, Normark SF, Harding CV. 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature.* 361:359-362.
268. Pierre P., S.J. Turley, E. Gatti, M. Hull, J. Meltzer, A. Mirza, K. Inaba, R.M. Steinman and I. Mellman. 1997. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature.* 388:787-792.
269. Pines J., and T. Hunter. 1992. Cyclins A and B1 in the human cell cycle. *Ciba Found. Symp.* 170:187-196.
270. Pines, J. and T. Hunter. 1990. Human cyclin A is adenovirus E1A - associated protein p60 and behaves differently from cyclin B. *Nature* 346:760-763.
271. Pinet V., M.S. Malnati and E.O. Long. 1994. Two processing pathways for the MHC class II-restricted presentation of exogenous influenzae virus antigen. *J. Immunol.* 162:4852-4860.

272. Pirofski L., R. Lui, M. DeShaw, A.B. Kressel, and Z. Zhong. 1995. Analysis of human monoclonal antibodies elicited by vaccination with a *Cryptococcus neoformans* glucuronoxylomannan capsular polysaccharide vaccine. *Infect. Immun.* 63(8):3005-3014.
273. Pirofski L.A. and A. Casadevall. 1996. *Cryptococcus neoformans*: paradigm for the role of antibody immunity against fungi? *Zentralbl. Bakteriologie*. 284:475-495.
274. Portnoy D.A., P.S. Jacks, D.J. Hinrichs. 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. *J. Exp. Med.* 167:1459-1471.
275. Powderly, W.G., E.J. Keath, M. Sokol-Anderson, K. Robinson, D. Kitz, J.R. Little, and G. Kobayashi. 1992. Amphotericin B resistant *Cryptococcus neoformans* in a patient with AIDS. *Infect Dis Clin. Pract.* 1:314-316.
276. Prigozy T.I., P.A. Sieling, D. Clemmens, P.L. Stewart, S.M. Behar, S.A. Porcelli, M.B. Brenner, R.L. Modlin, and M. Kronenberg. 1997. The mannose receptor delivers lipoglycan antigens to endosomes for presentation to T cells by CD1b molecules. *Immunity*. 6:187-197.
277. Pure E., K. Inaba, M.T. Crowley, L. Tardelli, M.D. Witmer-Pack, G. Ruberti, G. Fathman and R.M. Steinman. 1990. Antigen processing by epidermal Langerhans cells correlations with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain. *J. Exp. Med.* 172:1459-x.
278. Puri J. and Y. Factorovich. 1988. Selective inhibition of antigen presentation to cloned T cells by protease inhibitors. *J. Immunol.* 141:3313-3317.
279. Puri J., P. Lonai and V. Friedman. 1986. Antigen-Ia interaction and the proteolytic processing of antigen: the structure of the antigen determines its restriction to the A or E molecule of the major histocompatibility complex. *Eur. J. Immunol.* 16:1093-1097.
280. Racoosin, E.L., and J.A. Swanson. 1993. Macropinosome maturation and fusion with tubular lysosomes in macrophages. *J. Cell. Biol.* 121:1011-1020.
281. Rawlings N.D. and A.J. Barrett. 1994. Structure of membrane glutamate carboxypeptidase. *Biochem. Biophys. Acta.* 1339:247-252.
282. Reed, J. C. and P. C. Nowell. 1988. Soluble inhibitors of T lymphocyte proliferation: Tools for dissecting Pathways of T cell activation. *Cell. Immunol.* 7:93-112.
283. Reed, J. C., J. D. Alpers, P. C. Nowell, and R. G. Hoover. 1986. Sequential expression of protooncogenes during lectin-stimulated mitogenesis of normal human lymphocytes. *Proc. Natl. Acad. Sci. USA* 83:3982-3986.

- 284.Reid P.A. and C. Watts 1990. Cycling of cell-surface MHC glycoproteins through primaquine-sensitive intracellular compartments. *Nature*. 346:655-657.
- 285.Reid P.A. and C. Watts. 1992. Constitutive endocytosis and recycling of major histocompatibility complex class II glycoproteins in human B-lymphoblastoid cells. *Immunol*. 77:539-542.
- 286.Reis e Sousa C., and R.N. Germain. 1995. Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigen by a subset of cells engaged in phagocytosis. *J. Exp. Med*. 182:841-851.
- 287.Reis e Sousa C., P.D. Stahl, and J.M. Austyn. 1993. Phagocytosis of antigens by Langerhans cells *in vitro*. *J. Exp. Med*. 173:509-519.
- 288.Reiss E., E.H. White, R. Cherniak, and J.E. Dix. 1986. Ultrastructure of acapsular mutant *Cryptococcus neoformans* cap 67 and monosaccharide composition of cell extracts. *Mycopathologia*. 93:45-54.
- 289.Rescigno, M. C. Winzler, D. Delia, C. Mutinit, M. Lutz and P. Ricciardi-Castagnoli. 1997. Dendritic cell maturation is required for initiation of the immune response. *J. Leuk. Biol*. 61:415-421.
- 290.Retini C., A. Vecchiarelli, C. Monari, C. Tascini, R. Bistoni, and T.R. Kozel. 1996. Capsular polysaccharide of *Cryptococcus neoformans* induces proinflammatory cytokine release by human neutrophils. *Infect. Immun*. 64(8):2897-2903.
- 291.Retini C., A. Vecchiarelli, C. Monari, F. Bisoni, and T.R. Kozel. 1998. Encapsulation of *Cryptococcus neoformans* with glucuronylmannan inhibits the antigen-presenting capacity of monocytes. *Infect. Immun*. 66(2):664-669.
- 292.Rey-Millet C., C. L. Villiers, F.M. Gabert, S. Chesne, and M.G. Colomb. 1994. C3b covalently associated to tetanus toxin modulates TT processing and presentation by U937 cells. *Mol. Immunol*. 17:1321-1327.
- 293.Rhodes J.M. and A.B. Anderson. 1993. Role of cathepsin D in the degradation of human serum albumin by peritoneal macrophages and veiled cells in antigen presentation. *Immunol. Lett*. 37:103-110.
- 294.Roake J.A., A.S. Rao, P.J. Morris, C.P. Larsen, D.R. Hankins, and J.M. Austyn. 1995. Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J. Exp. Med*. 181:2237-2247.
- 295.Roche P.A. and P. Cresswell. 1990. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature*. 345:615-618.

296. Rock K.L., L. Rothstein, S. Gamble, and C. Fleischacker. 1993. Characterization of antigen-presenting cells that present exogenous antigens in association with class I MHC molecules. *J. Immunol.* 150(2):438-446.
297. Romani N. S. Gruner, D. Brang, E. Kampgen, A. Lenz, B. Trockenbacher, G. Konwalinka, P.O. Fritsch, R.M. Steinman, and G. Schuler. 1994. Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* 180:83-93.
298. Roth R. and H.L. Spiegelberg. 1996. Activation of cloned human CD4⁺ Th1 and Th2 cells by blood dendritic cells. *Scand. J. Immunol.* 43(6):646-651.
299. Salgame P., J.S. Abrams, C. Clayberger, H. Goldstein, R.L. Modlin, and B.R. Bloom. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science.* 254:279-282.
300. Salkowski C.A. and E. Balish. 1991. A monoclonal antibody to gamma interferon blocks augmentation of natural killer cell activity induced during systemic cryptococcosis. *Infect. Immun.* 59:486-493.
301. Sallusto F. and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α . *J. Exp. Med.* 179:1109-1118.
302. Sallusto F., M. Cella, C. Danieli, and A. Lanzavecchia. 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J. Exp. Med.* 182:389-400.
303. Sanfelice F. 1894. Contributo alla morfologia e biologia dei blastomiceti che si sviluppano nei succhi di alcuni frutti. *Ann. Igiene.* 4:463-495.
304. Sarosi G.A., J.D. Parker, I.L. Doto, and F.E. Tosh. 1969. Amphotericin B in cryptococcal meningitis: long-term results of treatment. *Ann Int Med.* 71:1079-87.
305. Saxon A., J. Feldhaus, and R. A. Robins. 1976. Single step separation of human T and B cells using AET treated SRBC Rosettes. *J. Immunol. Meth.* 12:285-288.
306. Scheicher C., M. Mehlig, H. Dienes and K. Reske. 1995. Uptake of microparticle-adsorbed protein antigen by bone marrow-derived dendritic cells results in up-regulation of interleukin-1 α and interleukin-12 p40/p45 and triggers prolonged, efficient antigen presentation. *Eur. J. Immunol.* 25: 1566-1572.

307. Scherer M.T., B.M. Chan, F. Ria, J.A. Smith, D.L. Perkins, and M.L. Gefter. 1989. Control of cellular and humoral immune responses by peptides containing T-cell epitopes. *Cold. Spr. Harb. Symp. Quant. Biol.* 54Pt 1:497-504.
308. Schimpff, S. C., and J. E. Bennett. 1975. Abnormalities in cell-mediated immunity in patients with *Cryptococcus neoformans* infection. *J. Allergy Clin. Immunol.* 55:430-41.
309. Schlesinger, L.S. 1993. Macrophages phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. *J. Immunol.* 150:2920-2930.
310. Schmitt-Verhulst, A., A. Guimezanes, C. Boyer, M. Poenie, R. Tsien, M. Buferne, C. Hua and L. Leserman. 1987. Pleiotropic loss of activation pathways in a T-cell receptor α -chain deletion variant of a cytolytic T-cell clone. *Nature* 325:628-631.
311. Schneider S.C. and E.E. Sercarz. 1997. Antigen processing differences among APC. *Hum. Immunol.* 54:148-158.
312. Schrezenmeier, H. and B. Fleischer. 1988. A regulatory role for the CD4 and CD8 molecules in T cell activation. *J. Immunol* 142:398-403.
313. Seaton R.A., J.P. Wembri, P. Armstrong, J. Ombiga, S. Naraqui and I. Kevau. 1996. Symptomatic human immunodeficiency virus (HIV) infection in Papua New Guinea. *Aust. New Zeal. J. Med.* 26(6):783-8.
314. Seder R.A., J.L. Boulay, F. Finkelman, S. Barbier, S.Z. Ben-Sasson, B. Le Gros, and W.E. Paul. 1992. CD8+ T cells can be primed *in vitro* to produce IL-4. *J. Immunol.* 148:1652-1656.
315. Selvan, R. S., P. S. Nagarkatti and M. Nagarkatti. 1990. Role of IL-2, IL-4 and IL-6 in the growth and differentiation of tumor-specific CD4+ T helper and CD8+ T cytotoxic cells. *Int. J. Cancer:* 45:1096-1104.
316. Sertl K., T. Takemura, E. Tschachler, V.J. Ferrans, M.A. Kaliner, and E.M. Shevach. 1996. Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma, and visceral pleura. *J. Exp. Med.* 163: 436-451.
317. Shenker, B. J. and S. Datar. 1995. *Fusobacterium nucleatum* inhibits human T-cell activation by arresting cells in the mid-G1 phase of the cell cycle. *Infect. Immun.* 63:4830.
318. Sherr C.J. 1993. Mammalian G1 cyclins. *Cell.* 73: 1059-065.

- 319.Small, J. M., T. G. Mitchell, and R. W. Wheat. 1986. Strain variation in composition and molecular size of the capsular polysaccharide of *Cryptococcus neoformans* serotype A. *Infect. Immun.* 54:735-41.
- 320.Smith K.A. 1988. Interleukin-2: inception, impact and implications. *Science.* 240:1169-1176.
- 321.Soreng K.M., D.A. Weber, H. Joshi, J.C. Moore, and P.E. Jensen. 1995. A role for microfilaments but not microtubules in processing of soluble antigens. *Cell. Immunol.* 166(1):25-34.
- 322.Spickard A. W.T. Butler, V. Andriole, V. and J.P. Utz. 1963. The improved prognosis of cryptococcal meningitis with amphotericin B therapy. *Ann. Int. Med.* 58:66-83.
- 323.Spies T., M. Breshnahan S. Bahrarn, D. Arnold, G. Blanck, E. Mellins, D. Pious, and R. DeMars. 1990. A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway. *Nature.* 348:744-747.
- 324.Spies T.M. and R. DeMars. 1991. Restored expression of major histocompatibility class I molecules by gene transfer of a putative peptide transporter. *Nature.* 351:323-324.
- 325.Spiropulu C., R.A. Eppard, E. Otteson, and T.R. Kozel. 1989. Antigenic variation within serotypes of *Cryptococcus neoformans* detected by monoclonal antibodies specific for the capsular polysaccharide. *Infect. Immun.* 57(10)3240-3242.
- 326.Sprent, J. and M. Schaefer. 1985. Properties of purified T cell subsets. I. *In vitro* responses to class I vs. class II H-2 alloantigens. *J. Exp. Med.* 162:2068-2088.
- 327.Stahl P.D. 1992. The mannose receptor and other macrophage lectins. *Curr. Opin. Immunol.* 4:49-52.
- 328.Stahl P.D. and R.A.B. Ezekowitz. 1998. The mannose receptor is a pattern recognition receptor involved in host defense. *Curr. Opin. Immunol.* 10:50-55.
- 329.Steinman R. 1991. The dendritic cell system and its role in immunogenicity. *Ann. Rev. Immunol.* 9:271-296.
- 330.Steinman R. M. and Z.A. Cohn. 1974. Identification of a novel cell type in peripheral lymphoid organs of mice. *JEM.* 139:380-397.
- 331.Steinman R.M. and J. Swanson. 1995. The endocytic activity of dendritic cells. *J. Exp. Med.* 283-288.

332. Stern J.B. and K.A. Smith. 1986. Interleukin-2 induction of T-cell G1 progression and c-myc expression. *Science*. 233:203-206.
333. Stevens, DA. 1991. Fungal infections in AIDS patients. *BCJP*. S71:11-21
334. Stoorvogel. W. 1993. Arguments in favour of endosome maturation. *Biochem. Soc. Trans.* 21:711-715.
335. Stossel H., F. Koch, E. Kampgen, P. Stoger, A. Lenz, C. Heufler, N. Romani, and G. Schuler. 1990. Disappearance of certain acidic organelles (endosomes and Langerhans cell granules) accompanies loss of antigen processing capacity upon culture of epidermal Langerhans cells. *J. Exp. Med.* 172:1472-1482.
336. Streicher H.Z., I.J., Berkower, M. Busch, F.R.N. Gurd, and J.A. Berzofsky. 1984. Antigen conformation determines processing requirements for T-Cell activation. *PNAS*. 81:6831-6835.
337. Sugar A.M. and C. Saunders. 1988. Oral fluconazole as suppressive therapy of disseminated cryptococcosis in patients with acquired immunodeficiency syndrome. *Am. J. Med.* 85:481-489.
338. Sugar, A.M., J.J. Stern, and B. Dupont. 1990. Overview: Treatment of cryptococcal meningitis. *Rev. Infect. Dis.* 12(Sup 3):S338-S348.
339. Sukroongreung S., C. Nilakul, O. Ruangsomboon, W. Chuakul, and B. Eampokalap. 1996. Serotypes of *Cryptococcus neoformans* isolated from patients prior to and during the AIDS era in Thailand. *Mycopath.* 135:75-78.
340. Svensson M., B. Stockinger, and M.J. Wick. 1997. Bone marrow-derived dendritic cells can process bacteria for MHC-I and MHC-II presentation to T cells. *J. Immunol.* 158(9):4229-4236.
341. Swanson J.A. and C. Watts. 1995. Macropinocytosis. *Trends. Cell. Biol.* 5:424-428.
342. Swanson J.A. and S. Baer. 1995. *Trends Cell. Biol.* 5:89-93.
343. Swoboda, R., E. Wecker, and A. Schimpl. 1987. Regulation of IL 2 expression in mitogen-activated murine T lymphocytes. *Immunobiology* 174:300-312.
344. Takahashi H, Cease KB, Perzofsky JA. Identification of proteases that process distinct epitopes on the same protein. *J. Immunol.* 1989;142:2221-2229.
345. Takemura R. and Z. Werb. 1984. Secretory products of macrophages and their physiological functions. *Am. J. Physiol.* 246(1 Pt 1):C-C9.

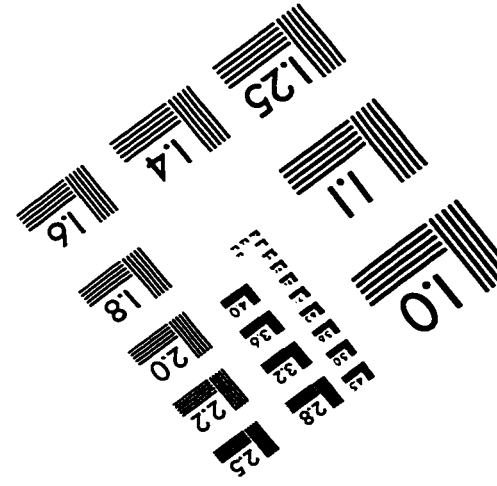
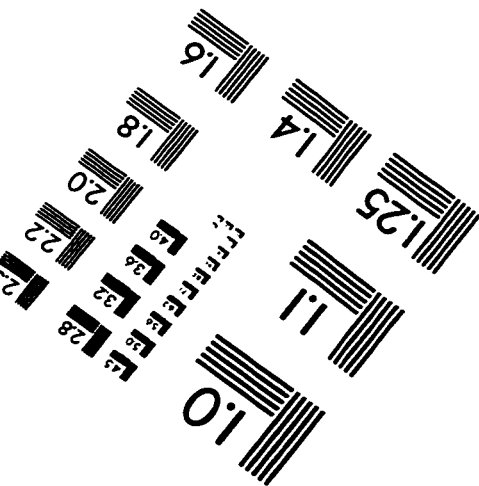
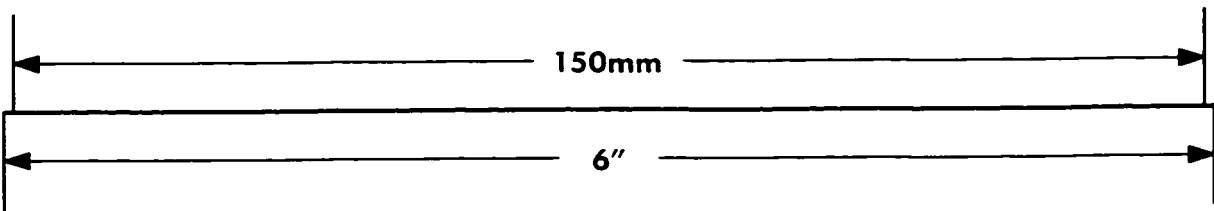
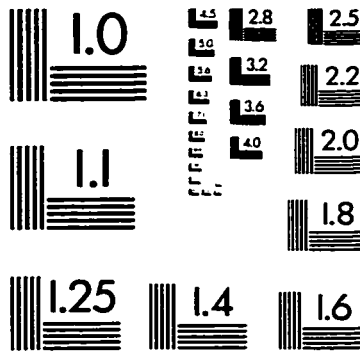
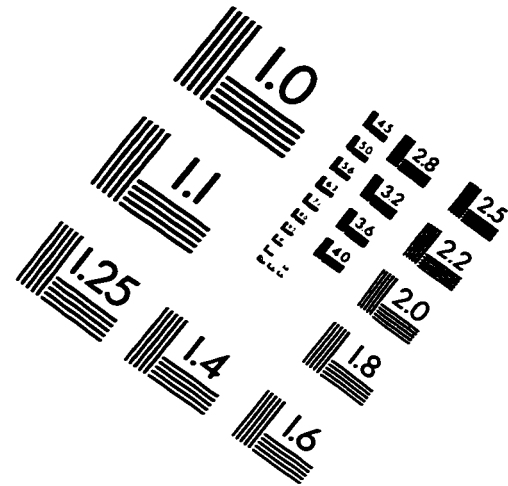
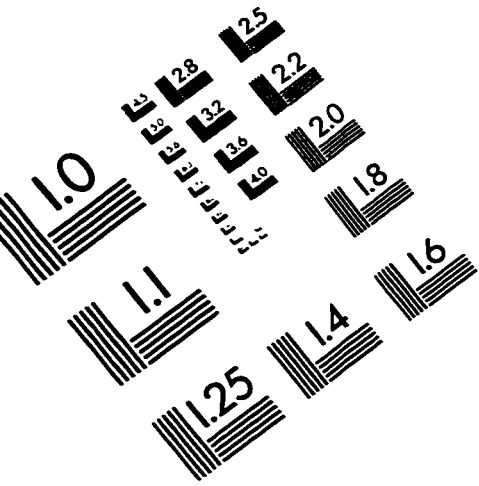
346. Tan M.C.A., A.M. Mommaas, J.W. Drijhout, R. Jordens, J.J. M. Onderwater, D. Verwoerd, A.A. Mulder, A.N. van der Heiden, T.H.M. Ottenhoff, M. Cella, A. Tulp, J.J. Neefjes, and F. Koning. 1997. Mannose receptor mediated uptake of antigens strongly enhances HLA-class II restricted antigen presentation by cultured dendritic cells. *Adv. Exp. Med. Biol.* 417:171-174.
347. Thiele D.L. and P.E. Lipsky. 1982. The accessory function of phagocytic cells in human T cell and B cell responses. *J Immunol.* 129: 1033.
348. Thompson C.B. 1995. Distinct roles for the costimulatory ligand B7-1 and B7-2 in T helper differentiation? *Cell.* 81:979-982.
349. Toews G.B., W.C. Vial, M.M. Dunn, P. Guzzetta, G. Nunez, P. Stastny, and M.F. Lipscomb. 1984. The accessory cell function of human alveolar macrophages in specific T cell proliferation. *J. Immunol.* 132:181-186.
350. Townsend A., and Bodmer H. (1989) Antigen recognition by class I- restricted T lymphocytes. *Annu Rev Immunol.* 7, 601-624.
351. Tsai, L., E. Lees, B. Haha, E. Harlow, and K. Riabowol. 1993. The cdk2 kinase is required for the G1 to S phase transition in mammalian cells. *Oncogene* 8:1593-1602.
352. Turner J., and Dockrell H.M. 1996. Stimulation of human peripheral blood mononuclear cells with live *Mycobacterium bovis* BCG activates cytolytic CD8+ T cells *in vitro*. *Immun.* 87, 339-342.
353. Unanue E.R. 1984. Antigen-presenting function of the macrophage. *Annu. Rev. Immunol.* 2:395-428.
354. Unanue E.R. and P.M. Allen. 1987. The basis for the immunoregulatory role of macrophages and other accessory cells. *Science.* 236:551-557.
355. Van Bleek G.M. and S.G. Nathenson. 1990. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2Kb molecule. *Nature.* 348:213-216.
356. Van Haarst J.M., H.C. Hoogsteden, H.J. de Wit, G.T. Verhoeven, C.E. Havenith and H.A. Drexhage. 1994. Dendritic cells and their precursors isolated from human bronchoalveolar lavage: immunocytologic and functional properties. *Am. J. Respir. Cell Mol. Biol.* 11:344-350.
357. van Ham, S.M., U. Gruneberg, G. Malcherek, I. Broker, A. Melms, and J. Trowsdale. 1996. Human histocompatibility leukocyte antigen (HLA)-DM edits peptides presented by HLA-DR according to their ligand binding motifs. *J. Exp. Med.* 184(5):2019-2024.

358. Van Voorhis W.C., L.S. Hair, R.M. Steinman, and G. Kaplan. 1982. Human Dendritic cells. Enrichment and characterization from peripheral blood. *J. Exp. Med.* 155:1172-1187.
359. Vanbreuseghem R., and M. Takashio. 1970. An atypical strain of *Cryptococcus neoformans* Vuillemini 1894. Part II *Cryptococcus neoformans var gatti var no.* *Ann Soc Belge Med trop.* 50:695-702.
360. Vartivarian S.E., G.H. Reyes, E.S. Jacobson, P.G. James, R. Cherniak, V.R. Mumaw and M.J. Tingler. 1989. Localization of mannoprotein in *Cryptococcus neoformans*. *J. Bacteriol.* 171:6850-6852.
361. Vecchiarelli A. M. Dottorini, D. Pietrella, C. Monari, C. Retini, T. Todisco, and F. Bistoni. 1994a. Role of human alveolar macrophages as antigen-presenting cells in *Cryptococcus neoformans* infection. *Am. J. Resp. Cell Mol. Biol.* 11:130-137.
362. Vecchiarelli A., C. Monari, C. Retini, D. Pietrella, B. Palazzetti, L. Pitzurra, and A. Casadevall. 1998a. *Cryptococcus neoformans* differently regulates B7-1(CD80) and B7-2(CD86) expression on human monocytes. *Eur. J. Immunol.* 28:114-121.
363. Vecchiarelli A., C. Retini, C. Monari, and A. Casadevall. 1998b. Specific antibody to *Cryptococcus neoformans* alters human leukocyte cytokine synthesis and promotes T-cell proliferation. *Infect. Immun.* 66(3):1244-1247.
364. Vecchiarelli A., C. Retini, C. Monari, C. Tascini, F. Bistoni, and T.R. Kozel. 1996. Purified capsular polysaccharide of *Cryptococcus neoformans* induces interleukin-10 secretion by human monocytes. *Infect Immun.* 64(7):2846-2849.
365. Vecchiarelli A., C. Retini, D. Pietrella, C. Monari, C. Tascini, T. Beccari, and T. R. Kozel. 1995. Downregulation by Cryptococcal polysaccharide of tumor necrosis factor alpha and interleukin-1b secretion from human monocytes. *Infect. and Immun.* 63(8). 2919-2923.
366. Vecchiarelli A., D. Pietrella, M. Dottorini, C. Monari, C. Retini, T. Todisco, and F. Bistoni., 1994b. Encapsulation of *Cryptococcus neoformans* regulates fungicidal activity and the antigen presentation process in human alveolar macrophages. *Clin. Exp. Immunol.* 98:217-22.
367. Vidard L., K.L. Rock and B. Benacerraf. 1991. The generation of immunogenic peptides can be selectively increased or decreased by proteolytic enzyme inhibitors. *J. Immunol.* 147(6):1786-1791.
368. Vidard L., K.L. Rock and B. Benacerraf. 1992. Heterogeneity in antigen processing by different types of antigen-presenting cells: effect of cell culture on antigen processing ability. *J. Immunol.* 149:1905-1911.

369. Vidard L., M. Kovacsovics-Bankowski, S.K. Kraeft, L.B. Chen, B. Benacerraf, and K.L. Rock. 1996. Analysis of MHC class II presentation of particulate antigens of B lymphocytes. *J. Immunol.* 156(8):2809-2818.
370. Wagner RP, Levitz SM, Tabuni A, Kornfeld H. 1992. HIV-1 envelope protein (gp120) inhibits the activity of human bronchoalveolar macrophages against *Cryptococcus neoformans*. *Am Rev Respir Disease.* 146:143-148.
371. Waldman, T. A. 1991. The interleukin-2 receptor. *J. Biol. Chem.* 266:2681-2684.
372. Weaver C.T., C. M. Hawrylowicz, and E.R. Unanue. 1988. T helper cell subsets require the expression of distinct costimulatory signals by antigen-presenting cells. *Proc Natl Acad Sci USA.* 85(21):8181-8185.
373. Weinberg P.B., S. Becker, D.L. Granger, and H.S. Koren. 1987. Growth inhibition of *Cryptococcus neoformans* by human alveolar macrophages. *Am Rev. Respir. Dis.* 136:1242-1247.
374. Wilson M.E., and R.D. Pearson. 1988. Roles of CR3 and mannose receptors in the attachment and ingestion of *Leishmania donovani* by human mononuclear phagocytes. *Infect. Immun.* 56(2):363-369.
375. Wilson, D. E., J. E. Bennett, and J. W. Bailey. 1968. Serologic Grouping of *Cryptococcus neoformans*. *Proc. Soc. Exp. Biol. Med.* 127:820-3.
376. Wong, H., W. D. Anderson, T. Cheng, and K. T. Riabowol. 1994. Monitoring mRNA expression by polymerase chain reaction: The primer-dropping method. *Anal. Biochem.* 223:251-258.
377. Yang Y., K. Fruh, J. Chambers, J.B. Waters, L. Wu, T. Spies, and P.A. Peterson. 1992. Major histocompatibility complex (MHC)-encoded HAM2 is necessary for antigenic peptide loading onto class I MHC molecules. *J. Biol. Chem.* 267:11669-x.
378. Young B.J. and T.R. Kozel. 1993. Effects of strain variation, serotype and structural modification on kinetics for activation and binding of C3 to *Cryptococcus neoformans*. *Infect. Immun.* 61:2966-2972.
379. Young J.W., P. Szabocs, and M.A.S. Moore. 1995. Identification of dendritic cell colony forming units among normal CD4+ bone marrow progenitors that are expanded by c-kit ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony stimulating factor. *J. Exp. Med.* 182:1111-1120.
380. Yuan R., A. Casadevall, G. Spira, and M.D. Scharff. 1995. Isotype switching from IgG3 to IgG1 converts a nonprotective murine antibody to *Cryptococcus neoformans* into a protective antibody. *J. Immunol.* 154:1810-1816.

381. Yuan R., A. Casadevall, J. Oh. and M.D. Scharff. 1997. T cells cooperate with passive antibody to modify *Cryptococcus neoformans* infection in mice. P.N.A.S. USA. 94:2483-2488.
382. Zeigler, K. and E.R. Unanue 1981. Identification of a macrophage antigen-processing event required for I-region-restricted antigen presentation to T lymphocytes. J Immunol. 127(5): 1869-1875.
383. Zhong G., P. Romagnoli, and R.N. Germain. 1997. Related leucine-based cytoplasmic targeting signals in invariant chain and major histocompatibility complex class II molecules control endocytic presentation of distinct determinants in a single protein. J. Exp. Med. 185:429-438.
384. Zhong Z. and L. Pirofski, 1996. Opsonization of *Cryptococcus neoformans* by human anticryptococcal glucuronoxylomannan antibodies. Infect. Immun. 64(9):3446-3450.
385. Zhong Z., and L.A. Pirofski. 1998. Antifungal activity of a human antiglucouronoxylomannan antibody. Clin. Diagn. Lab. Immunol. 5(1):58-64.
386. Zhou L. and T.F. Tedder. 1995. Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. J. Immunol. 154:3821-3835.
387. Ziegler H.K., and E.R. Unanue. 1982. Decrease in macrophage catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T-cells. Proc Natl Acad Sci USA;79:175-181.
388. Zuger, A., E. Louie, R. S. Holzman, M. S. Simberkoff, and J. J. Rahal. 1986. Cryptococcal disease in patients with the acquired immune deficiency syndrome. Ann. Int. Med. 104:234-240.

IMAGE EVALUATION TEST TARGET (QA-3)



APPLIED IMAGE, Inc
 1653 East Main Street
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