Cryptococcus neoformans

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Kroesje

Jij kleurde de dag
met je betoverende lach
liet de wereld stralen
en ieder die het zag

Zo geliefd door velen
flamboyant en energiek
bereid alles te delen
zij aan zij met Annemiek

Wat een leegte laat je achter
wat een troosteloos verdriet
maar je leeft voort in gedachten
en in gedachten sterf je niet

(J.W. Peterse mei 2000)

Voor Robert Kroes
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1. CRYPTOCOCCOSIS

1.1 History of *Cryptococcus neoformans*

Cryptococcus neoformans is an encapsulated yeast-like fungus, which can cause a systemic infection (cryptococcosis), mainly in patients with impaired cell mediated immunity. The organism was identified as a human pathogen in 1894 by the pathologist Busse, and the surgeon Buschke (1). They independently cultured the same fungus from lesions of the tibia and cutaneous lesions, respectively, of a 31-year old woman. In the same year, *C. neoformans* was isolated from peach juice by the Italian Sanfelice, who later proved its pathogenicity in laboratory animals (2).

Until 1950 many case reports were published on clinical infection with *C. neoformans*, including animal experiments with the organism isolated from these patients (3). Infection in the brain, lungs, kidneys and many other organs were described. Pathologist reported gelatinous masses and chronic granulomatous and tumor-like lesions (4) as well as relative absence of polymorphonuclear infiltrates (5). These different appearances of the yeast caused an on-going confusion with regard to the correct nomenclature. For example, *Saccharomyces hominis, Cryptococcus hominis, Torula histolytica, Debaryomyces hominis* were all early names for the pathogen. Around 1950 the name *C. neoformans* was established and two varieties were recognized: var. *neoformans* and var. *gattii*. Currently we recognize 3 varieties: *grubii* (serotype A), *gattii* (serotype B, C) and *neoformans* (serotype D). Most likely, var. *gattii* will be renamed as a novel species and called *C. bacillisporus* (6). The name of the teleomorphic form of *C. neoformans var. neoformans* is *Filobasidiella neoformans* (7).

From 1981 on the incidence of *C. neoformans*-infected patients has increased dramatically as a result of the ongoing AIDS epidemic. An estimated 36 million people worldwide are currently living with HIV, and some 20 million people have already died, giving a cumulative total number of HIV infections of 56 million (8). There were 5.3 million new HIV infections globally in 2000, and there is a clear potential for further massive spread. In most western countries 5-10% of HIV infected patients will develop cryptococcosis (9). In 1991 over 1200 cases of cryptococcal meningitis were diagnosed in New York City (10). Recently a decrease in incidence in these countries has been described, probably due to the introduction of HAART (Highly Active Antiretroviral Therapy) for treatment of HIV-infection (11). In sub-Saharan Africa, 10-15% of HIV infected patients will develop cryptococcosis (12). In Zimbabwe, however, cryptococcosis constitutes in 88% of HIV-infected patients the AIDS-defining illness, where it currently represents the most important cause of meningitis in adults (13;14).

Before 1950 untreated cryptococcal meningitis was, with some sporadic exceptions, in 100% of cases fatal (15). With the introduction of Amphotericin B in the late

1950's the first effective therapy against cryptococcosis was discovered (16) and death rates dropped to 10-25% of patients during treatment or in the immediate follow-up period (17;18). Due to the high prices of currently available antimycotics, we have unfortunately been able to observe the natural history of untreated HIV and cryptococcal meningitis in sub-Saharan countries. In Zimbabwe, for example median survival time from diagnosis to death is 14 days and only 22% of patients survived for more then 30 days without treatment (13). In Malawi, a median survival time after diagnosis is 4 days (19).

1.2 Clinical manifestation

Cryptococcosis, a systemic infection with *C. neoformans*, is mostly seen in patients who have a compromised immune system. Most important predisposing factors for cryptococcosis are HIV infection, use of immunosuppressive drugs, organ and bone marrow transplantation, chronic leukemias and lymphomas. The severity of clinical manifestation depends on the immune status of the host. In patients with AIDS, as compared to immunocompetent patients, the fungal burden is usually higher, and more extraneural sites of infection are present. In addition, relapse rates are higher. Time between first presentation and diagnosis is significantly longer in non-HIV infected patients (described in **chapter 2**). Cryptococcal meningitis (infection of the subarachnoid space) and meningoencephalitis (infection of both the subarachnoid space and the brain parenchyma) are the most frequently encountered life-threatening manifestations of cryptococcosis. Patients usually present with symptoms like headache, fever, nausea/vomiting, seizures, altered mentation, vision changes, and/or cranial nerve paresis which may have been already existing for 2 to 4 weeks before presentation. Physical examination can reveal signs such as papilledema and cranial nerve paresis. Nuchal rigidity does occur in 28% of HIV infected patients, and in 14% of non-HIV infected patients (**chapter 2**).

Pulmonary cryptococcosis is the second most relevant site of infection for *C. neoformans*. Clinical course depends on immune status of the host. *C. neoformans* can cause pneumonia in immunocompetent host. Symptoms include cough (54%), chest pain (46%), increased sputum production (32%), weight loss and fever (26%) (20). Immunocompromised (either HIV or non-HIV infected) patients with cryptococcal pneumonia generally have a more rapid clinical course and a greater tendency to disseminate their infection then immunocompetent patients (21;22).

Radiographically, cryptococcal pneumonia in the normal host may present with well-defined, non-calcified single or multiple lung nodules (23). Pulmonary cryptococcosis is diagnosed through antigen detection and culture of expectorated sputum, BAL (broncho alveolar lavage), transbronchial lung biopsy or needle aspiration. Serum and cerebrospinal fluid (CSF) analysis for antigen and cryptococcal culture should be performed to assess dissemination.

C. neoformans involvement of skin, eyes, genitourinary tract, bone and joints, muscle, heart, gastrointestinal tract, breast, lymph nodes, thyroid, adrenal gland head and neck has been described. Most of these cases should be considered as manifestations of disseminated cryptococcosis . It is currently an accepted practice to exclude presence of cryptococci in the brain by examination the CSF of all patients in whom the yeast has been isolated from another body site and who have defined risk factors for dissemination (see before) even if they are asymptomatic. Investigation of disseminated cryptococcosis should include CSF and blood culture as well as serological tests.

Microscopically, encapsulated *C. neoformans* can be detected in specimens of CSF or other host fluids dissolved in Indian ink preparations (24). Indian ink examination is a rapid test that can often deliver an immediate diagnosis within minutes after lumbar puncture. *C. neoformans* can be cultured from CSF, blood, sputum, urine and other specimens. Furthermore, latex agglutination tests for cryptococcal antigen (capsule) in body fluids are available (25). These tests are approximately 95% sensitive and 95% specific for identification of invasive cryptococcosis (26). Histopathologically, *C. neoformans* can be relatively easy identified in tissue because of its prominent capsule.

1.3 Management of cryptococcal disease

The choice of treatment for disease caused by *C. neoformans* depends on both the anatomic sites of involvement and the host's immune status. Recently, guidelines for the management of cryptococcal disease were published by M.S. Saag et al. (27). For immunocompetent hosts with isolated pulmonary disease, careful observation may be warranted; in case of symptomatic infection, the indicated treatment is fluconazole, 200-400 mg/day for 3-6 months. This treatment is also recommended for individuals with non-CNS-isolated cryptococcemia, a positive serum cryptococcal antigen titer >1:8, urinary tract or cutaneous disease. For patients with more severe disease, treatment with amphotericin B (0.5-1 mg/kg/d) may be necessary for 6-10 weeks. For otherwise healthy hosts with CNS disease, standard therapy consists of amphotericin B 0.7-1 mg/kg/d, plus flucytosine, 100 mg/kg/d, for 6-10 weeks. Fluconazole "consolidation" therapy may be continued for as long as 6-12 months, depending on the clinical status of the patient. HIV-negative, immunocompromised hosts should be treated in the same fashion as those with CNS disease, regardless of the site of involvement.

For those patients with HIV who present with isolated pulmonary or urinary tract disease, fluconazole at 200-400 mg/d is indicated, lifelong (see later). For patients with more severe disease, a combination of fluconazole (400 mg/d) plus flucytosine, (100-150 mg/kg/d) may be used for 10 weeks, followed by fluconazole maintenance therapy. Among patients with HIV infection and cryptococal meningitis, induction

therapy with amphotericin B 0.7-1 mg/kg/d, plus flucytosine, 100 mg/kg/d, for 2 weeks followed by fluconazole (400 mg/d) for a minimum of 10 weeks is the treatment of choice. After 10 weeks of therapy, the fluconazole dosage may be reduced to 200 mg/d, depending on the patient's clinical status.

Secondary prophylaxis against *C. neoformans* used to be indicated in all HIV infected patients with a history of cryptococcal disease. However, the introduction of HAART has dramatically changed the course of HIV infection (28). The improvements in immunological function, as the result of HAART, made it possible to stop primary and secondary prophylaxes against various opportunistic infections (29;30). Very recently, two studies suggesting that it is safe to stop secondary prophylaxis against *C. neoformans* in patients responding to HAART (increase in CD4 cell count to >100 cells/mm^3), without relapse of their cryptococcal disease have been published (31;32).

2. *C. NEOFORMANS* AND HOST RESPONSE

2.1 Innate host defense in the lung

C. neoformans is a worldwide occurring free-living organism that can survive in a variety of environmental niches. It has been isolated from pigeon and other avian excreta, fruits and soils (33). In contrast to bacteria, eukaryotic pathogens are generally not passed from person to person, and their evolution has not necessarily attained specific abilities to infect or invade the human host. A human fungal infection is generally the result of an accidental encounter during the life cycle of the fungus. Virulence genes and factors, in nature necessary for the organism to survive under various circumstances, are used to overcome the natural defenses of the host. A number of genes that allow the organism to grow and survive in the mammalian host have been defined.

Several lines of evidence suggest that human cryptococcosis result from inhalation of either desiccated, poorly encapsulated yeast forms, or basidiospores (34-36). These poorly encapsulated strains have a diameter of about 2 to 5 μm (37) and can penetrate the alveoli if not expelled through respiratory epithelia. In the alveolar spaces *C. neoformans* is initially confronted by alveolar macrophages (38), which play a central role in host defense against *C. neoformans*. Macrophages are able to bind, ingest and, with appropriate stimulation, kill yeast cells (39;40). Macrophage phagocytosis of *C. neoformans* can occur through either antibody (41), and complement receptors (42) (serum-dependent-phagocytosis) or mannose (39), and β-glucan (43) receptors (serum-independent-phagocytosis). In the alveoli primitive opsonins, termed collectins, are present which contribute to innate resistance against inhaled microorganisms (44-47).

Collectins are humoral lectins found in mammals and birds. They belong to the animal C-type lectin superfamily characterized by a carbohydrate recognition domain (CRD), which binds ligands in a Ca^{2+}-dependent manner, plus a collagen tail involved in their biological function (48). They are related structurally and functionally to the first component of the classical complement pathway, C1q, and serve important roles in innate immunity through opsonization and complement activation. The lectin domain binds carbohydrates on microorganisms, while the collagenous regions are ligands for the collectin receptor (C1q receptor) on phagocytes and also mediate C1q-independent activation of the classical complement pathway (49). The pulmonary surfactant proteins A (SP-A) and SP-D, as well as the serum collectins mannose-binding lectin, conglutinen and CL-43 have been identified (50). The serum collectin human mannose binding lectin is involved in TNF-α production by human monocytes stimulated with mannoprotein, one of the capsule components of *C. neoformans* (51). One of the pulmonary collectins, SP-D, has been described to agglutinate acapsular *C. neoformans* (52). The role of SP-D and SP-A in serum-independent phagocytosis of *C. neoformans* is described in **chapter 4**. Serum-free phagocytosis of acapsular *C. neoformans* by macrophages induces a range of proinflammatory cytokines, which may stimulate an effective cell mediated immune response before the yeasts are able to grow and synthesize appreciable quantities of capsule, which is known to undermine host defense (see later). Also, human alveolar macrophages containing phagocytosed *C. neoformans* can induce proliferation of autologous T lymphocytes, suggesting their role as antigen-presenting cells in cryptococcal infection (53).

2.2 Cell mediated immunity

2.2.1 Delayed type hypersensitivity

Full protection against *C. neoformans* is dependent on multiple factors, which include innate and adaptive immune defenses. The factors that stand out as being crucial are the functional CD^{4+} T helper cells and the ability of the host to mount a cell mediated immune response. The importance of cell-mediated immunity (CMI) in protection against *C. neoformans* has been firmly established in both animal experiments and patients (54-56). In absence of CD^{4+} T cells, patients with disseminated cryptococcosis are not able to clear the organism completely even with the best available antifungal therapy, and must be put on maintenance antifungal therapy for life (38) (see before). Evidence that CMI response induced by low dose infection with *C. neoformans* is protective has come from studies with nude mice (54). After infection, heterozygous nu/+ mice developed an anticryptococcal delayed type hypersensitivity (DTH) response and concomitantly the numbers of cryptococcal Colony Forming Units (CFU) in tissues were reduced. T-cell-deficient nude (nu/nu) mice did not develop an anticryptococcal CMI response but did make high levels of anticryptococcal antibodies and showed no signs of controlling the infection (54).

Positive DTH responses not only indicate the presence in the body of activated T cells, but also confirm that other components in the CMI cascade such as cytokines, chemokines, adhesion molecules, chemokine receptors and leukocyte migration are functioning.

The leukocyte infiltrate after intratracheal inoculation of *C. neoformans* into susceptible mice (like CBA/J mice) consists of macrophages, lymphocytes (CD^{4+} T cells, CD^{8+} T cells, B cells and NK cells), neutrophils and eosinophils (57;58). Activated macrophages appear to be the most important fungicidal effector cell (40;59). Thus, the recruitment and activation of phagocytes is an important component of CMI during cryptococcal infection. Mechanisms by which the host defense cells are attracted to the site of infection are complex. They involve local production of chemotactic factors that promote directed migration of phagocytes.

2.2.2 Chemokines

Specific substances termed chemotactic factors trigger directed locomotion of phagocytes towards the actual site of infection (chemokinesis). The phagocytes carry several receptors on their cell surface by which they are able to recognize these chemotactic factors. Chemoattractants are either secreted by activated host cells, or produced by complement activation or by the invading microorganism itself. They form gradients, which determine the direction of phagocyte migration. Well known chemoattractants for phagocytes are platelet-activating factor (PAF), arachidonate metabolite leukotrine B_4 (LTB_4), complement fragment C5a, bacterial derived formylated peptides like N-formyl-methionyl-leucyl-phenylalanine (fMLP) and chemokines like IL-8 and MCP. The two most important chemokine families are subdivided based on the position of the first two conserved cysteine residues: C-C chemokines (two adjacent cysteine residues) and the C-X-C chemokines (two cysteine residues separated by one aminoacid) (60;61). The C-C chemokines include MCP-1, MCP-2, MCP-3, RANTES, MIP-1α and MIP-1β, and are predominantly chemotactic for mononuclear cells. The C-X-C chemokines include IL-8, MIP2, and PF4, attract mainly neutrophils. Binding of chemotactic factors to their specific receptors activates multiple intracellular signaling pathways that regulate the intracellular machinery necessary to propel the cell in its chosen direction. The C-C chemokine MCP-1 is involved in clearance of pulmonary cryptococcal infection, and plays a critical role in the T cell-dependent immune response to *C. neoformans* (62). MIP-1α production is required during the efferent phase of pulmonary CMI for the recruitment of macrophages and neutrophils to the site of *C. neoformans* infection (63). Data of Huffnagle *et al*. (62;63) support the hypothesis that MCP-1 plays an important role in the initial recruitment of leukocytes (T lymphocytes and a small number of monocytes and neutrophils) that produce MIP-1α, which, in turn, mediates the bulk of monocytes

and neutrophil recruitment into the lungs. Furthermore, neutralization of MIP-1α reduces both TNF-α and IL-6 levels in *C. neoformans* infected mice (63).

2.2.3 Cytokines

Peripheral blood mononuclear cells (PBMC) and neutrophils have been shown to produce proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 when stimulated with *C. neoformans* or their cell wall components (51;64;65). The presence of certain cytokines during the induction phase of a CMI response also determines whether Th1 cells or Th2 cells predominate as activated T cells (66). Typically, the presence of IL-12, IL-18 or IFN-γ ensures that Th1 cells develop, whereas the presence of IL-10 and/or IL-4 directs the response to a Th2 response. Production of TNF-α is required for the development of protective T cell immunity to *C. neoformans* (67). The development of a protective Th1 type CMI and the production of Th1- and macrophage-activating cytokines TNF-α, IL1β, IL-12, IFN-γ, and GM-CSF are required to eradicate the infection (68), control cryptococcal dissemination from the lungs, and eliminate subsequent invasion in the brain (69). Phagocytes like macrophages, monocytes, and PBMC stimulated with pro-inflammatory cytokines show in general enhanced phagocytosis and fungicidal activity (70). In **chapter 5** cytokines, which are produced by PBMC of healthy donors stimulated with *C. neoformans* and its capsular components, have been studied.

2.3 Dissemination to the brain compartment

In cases of dissemination from the lungs, cryptococci are able to escape local (impaired) innate and adaptive immunity and gain access to the bloodstream. In the vascular compartment, polymorphonuclear cells and monocytes are present. These cells have demonstrated effective in vitro killing, especially in the presence of opsonins, like complement (71;72). *C. neoformans* has a unique, partly unexplained, predilection for the brain. The yeast produces a unique phenol oxidase that converts a variety of hydroxybenzoic substrates, including catecholamines (e.g. norepinephrine and dopamine), into melanin, which impart a dark color to colonies (73). Melanin can function as an antioxidant, which may protect *C. neoformans* from oxidative host defenses (74). The wide availability of catecholamines in the brain could be a factor for the neurotropic features of the yeast.

In order to produce infection in the brain yeast cells must cross the blood brain barrier. Endothelial cells are able to phagocytose acapsular *C. neoformans* through a serum-dependent process (75), however no direct killing activity has been demonstrated in vitro (76). These observations suggest that endothelial cells may take up poorly encapsulated forms and that this phenomenon may contribute to *C. neoformans* dissemination to the brain compartment (75). After arriving in the brain, biosynthesis routes leading to capsule formation are strongly up-regulated. Brain

cryptococci are characterized by the presence of huge capsules and high levels of capsular products are shed into the vascular compartment.

In an autopsy series of 27 patients with cryptococcal meningoencephalitis significant differences in neuropathologic inflammatory response between AIDS and non-AIDS infected patients were found (77). Most non-AIDS patients had granulomas, supporting a role for CMI, whereas AIDS patients did not show granulomatous inflammation. The principal reactive cells in cryptococcal meningoencephalitis in AIDS patients were brain macrophages and microglia. In order to investigate if chemokines play a role in the recruitment of these cells, the induction of MIP-1α, MIP1-β and RANTES by HIV and non-HIV infected macrophages stimulated by *C. neoformans* was investigated in **chapter 6**.

In HIV-infected patients with cryptococcal meningitis cytokine profiles in CSF were analyzed and showed high levels of the C-X-C chemokine, interleukin 8 (IL-8) (78). *C. neoformans* products such as GXM (see later) can directly induce IL-8 production by isolated microglial cells in culture (79). IL-8 is a chemotactic cytokine, which partially mediates the adhesion of neutrophils to the endothelium and their migration through the vascular wall. Despite the high levels of IL-8 in CSF of infected patients, no leucocytosis was observed (78). Cryptococcal polysaccharides are generally considered to mediate inhibition of neutrophil extravasation (80).

3. POLYSACCHARIDE CAPSULE

3.1 Chemical composition

A distinctive feature of *C. neoformans* relative to other medically important yeasts is the ability to produce an extracellular polysaccharide capsule. In nature it may protect the yeast from desiccation or reduce its ability to be ingested and destroyed by soil amoebae (81). In the pathogenesis of cryptococcosis the capsule has been shown to be a virulence factor (82;83). Several genes necessary for capsule formation CAP59, CAP64, CAP60, and CAP10 have been identified (82;84). In experimental cryptococcosis, capsule-free isolates are less virulent than their encapsulated wild-type cells (85). An encapsulated strain created by complementation of the CAP64 mutation produced fatal infection of mice within 25 days, while the CAP64 acapsular strain was avirulent (82). A number of effects of cryptococcal capsule polysaccharides, which may contribute to enhanced virulence, have been described. The capsule inhibits phagocytosis of *C. neoformans* by macrophages, monocytes, and neutrophils (86). The capsule does not directly modulate phagocytic function, but instead, presents a surface that is not recognized by phagocytes (87). Furthermore, interference with antigen presentation by the cryptococcal polysaccharides has been described (88).

The capsule can vary in size between <1 μm and >50 μm, depending on growing conditions. The capsule consists for 88% of glucuronoxylomannan (GXM), for 10% of galactoxylomannan (GalXM) and for 2% of mannoproteins (MP) (89). GXM is composed of a α-1,3-linked polymannose backbone with β-linked monomeric branches of xylose and glucuronic acid. The four described serotypes A, B, C and D are discriminated on the base of differences in GXM structure. The different serotypes have a common core-repeating unit, but differ in the degree of mannosyl substitution and the molar ratios of mannose, xylose, and glucuronic acid (90). GalXM is composed of a backbone of α (1→6) linked galactose substituted with small side chains consisting of mannose, xylose and galactose (91). For individual strains there are differences in sugar composition in the GalXM, indicating that this polysaccharide is structurally heterogeneous (92). MP and GalXM often fractionate together in polysaccharide preparations. When GalXM-containing material is further fractionated on a concanavalin A affinity chromatography column, three peeks containing GalXM, MP1/MP2 and MP4 are observed. MP is proposed to be consisting of a protein backbone heavily substituted by short oligosaccharides mainly containing mannose although significant amounts of galactose and xylose are present (91). MP4 consists of 20% protein (predominant amino acids are serine, threonine, and alanine) and sugar moieties (xylose-mannose-galactose in a 1 to 7.7 to 0.8 ratio) (91).

3.2 Interference with neutrophil migration

The circulatory and migratory properties of leukocytes have evolved to allow efficient surveillance of tissues for infectious pathogens and rapid accumulation at sites of injury and infection. It is currently believed that leukocytes leave the circulation by first adhering to activated endothelial cells, followed by migration through the interendothelial junctions (93). Localized inflammation results in dilatation of vessels and diminished blood flow, allowing the transient adherence of PMN to endothelial cells by selectin-mediated tethering and rolling along the vessel wall (94;95). CD62L-mediated rolling precedes a further functional up-regulation of PMN following exposure to pro-inflammatory cytokines and chemoattractants (96), resulting in firmer, integrin-mediated adherence. Up-regulation of integrins is accompanied by shedding of CD62L from the cell surface (94;96). Further stimulation of PMN phenotypically high in CD11b/CD18 (CR3, Mac-1) initiates transendothelial migration via an interaction of Mac-1 with its counterreceptor ICAM-1 (97). Finally, chemotactic gradients guide migrating PMN to the site of infection. Patients who are genetically deficient in the leukocyte integrins have been described. Neutrophils in these leukocyte adhesion deficiency I (LAD-I) patients fail to cross the endothelium and accumulate at inflammatory sites and in vitro are deficient in binding to and migrating across resting or activated endothelial monolayers (98).

The potency of *C. neoformans* culture supernatant to inhibit leukocyte migration was recognized almost half a century ago (99). Disseminated cryptococcosis is characterized by the presence of high levels of capsular antigens GXM and GalXM in the CSF and serum of affected patients (100;101). GXM titers in both serum and CSF from AIDS patients can reach levels up to 20 mg/ml (102). Despite the elevated IL-8 CSF/serum ratio and the elevated serum levels of TNF-α and IL-1β, the CSF of patients with cryptococcal meningitis typically contains few mononuclear cells and virtually no PMN (78). GXM is capable of interfering with leukocyte migration towards potent chemoattractants, such as fMLP and C5a (80;103;104). Furthermore, GXM can induce the production of IL-8 by human microglia but inhibits neutrophil migration towards IL-8 (79). GXM sheds L-selectin from neutrophils (105), and can bind to CD18 adhesion molecules thereby blocking interaction of activated neutrophils with ligands on the endothelium (106). To investigate if GXM actually interferes with leukocyte migration in clinical cryptococcosis, we compared retrospectively the GXM titer in serum and CSF with the CSF leukocyte cell counts of 35 Dutch HIV-infected patients with culture-proven diagnosis of cryptococcal meningitis (**chapter 7**). Furthermore, GXM has been described to delay translocation of PMN across the blood-brain barrier in a rabbit bacterial meningitis model (107).

Therefore, the initial aim to further study was to investigate the molecular mechanism by which GXM prevents PMN migration toward chemoattractants. Surprisingly, we found that cryptococcal culture filtrate (CneF) from GXM-producing and GXM-nonproducing (ΔCneF) strains, both prevented PMN migration towards IL-8 and fMLP. These results seriously question the opinion that GXM is the sole cryptococcal component preventing extravasation. The finding that MP-4 was primarily responsible for the inhibition of PMN migration is described in **chapter 8**.

4. OUTLINE OF THIS THESIS

In **chapter 2** a survey of cryptococcosis cases in the Netherlands is described during a 14-year retrospective analysis of cases (1986-2000). In **chapter 3, 4, 5 and 6** we follow *C. neoformans* on its route through the human body.

Cryptococcosis is likely to occur via inhalation of small, acapsular *C. neoformans*. The inhaled particles are small enough to reach alveolar spaces. One of the initial steps in host defense, is the interaction between acapsular cryptococci and alveolar macrophages. In a laboratory environment, this interaction (binding and phagocytosis) is usually measured in tubes where both target (*C. neoformans*) and effector cells (for example alveolar macrophages) are in suspension. *In vitro*, however it is more likely that alveolar macrophages act as adherent cells. Therefore, in **chapter 3** a method is described to measure phagocytosis of *C. neoformans* by adherent effector cells. Serum-free phagocytosis of *C. neoformans* by alveolar macrophages may be an

important part of the innate immune response because in the lung high concentration of serum opsonins, like complement factors, might be missing. **Chapter 4** describes the influence of non-serum opsonins, surfactant protein A and D on phagocytosis of *C. neoformans* by a number of effector cells, including human alveolar macrophages. A next step in immune response against *C. neoformans* includes influx of leucocytes to the side of infection, including PMN and mononuclear cells. Furthermore, in disseminated infection, cryptococci are able to escape to the vascular compartment. **Chapter 5** describes the cytokine profile of PBMC stimulated with *C. neoformans* and analysis the components responsible. Finally, yeast cells will be able to reach the brain compartment. In AIDS related cryptococcal meningoencephalitis brain macrophages and microglia were identified as principal inflammatory cells. In **chapter 6** a study is described which analysis which chemokines are produced by HIV-infected macrophages after stimulation with *C. neoformans* or GXM. CSF of patients with AIDS related cryptococcal meningoencephalitis contains few neutrophils despite high levels of neutrophil chemoattractant IL-8. As mentioned before cryptococcal polysaccharides are thought to play a role in the interference with neutrophil migration. In **chapter 7** patient data were analyzed for their titers of GXM in serum and CSF, and the results were correlated to matching CSF-counts. In **chapter 8**, the role of another cryptococcal polysaccharide, MP4, in interfering with leukocyte migration, is investigated.

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CHAPTER 2

Epidemiology of Cryptococcosis in the Netherlands: A 12-year Survey (1986-1999) and the Effect of Highly Active Antiretroviral Therapy (HAART).

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SUMMARY

To describe prevalence, clinical features, and prognosis of cryptococcosis in the Netherlands we performed a retrospective analysis. Data on cryptococcosis were collected nationwide from January 1986-December 1999 through a national reference laboratory and 5 major microbiology laboratories. Clinical data were obtained by reviewing medical records. In 14 years, cryptococcosis was diagnosed in 268 patients. The increasing number of cryptococcosis cases from 1987 on initially paralleled the growing AIDS epidemic, 203 patients with cryptococcosis (76%) were infected with HIV. Introduction of Highly Active Antiretroviral Therapy (HAART) in 1996 was associated with a sharp decline in the incidence of cryptococcosis. In non- HIV-infected patients, there was a significant delay in diagnosis. Favorable clinical outcome was associated with initial treatment with amphotericin B plus flucytosine. The strongest adverse prognostic factor in multi variate analysis was altered consciousness at the time of presentation at the hospital ($P<0.05$).

This retrospective analysis of cryptococcosis cases demonstrates changes in the occurrence of cryptococcosis that are mainly caused by changes in the epidemiology of AIDS in the Netherlands and are temporally associated with the introduction of HAART.

Because untreated cryptococcosis in HIV-, and non-HIV-infected patients has a high mortality rate, fast recognition and diagnosis of this infection is important.

INTRODUCTION

Cryptococcosis is a systemic mycotic infection caused by the encapsulated yeast *Cryptococcus neoformans*, mainly occurring in patients with impaired cell mediated immunity (1).

The incidence of cryptococcosis has increased dramatically worldwide in the last decades, paralleling the rising numbers of susceptible immunocompromised patients. Most important predisposing factors for infection with *C. neoformans* are HIV infection, use of immunosuppressive drugs, organ and bone marrow transplantation, chronic leukemia and lymphomas. In most western countries 5-10% of HIV infected patients will develop cryptococcosis (2). In sub-Saharan Africa 10-15 % of HIV infected patients become infected with *C. neoformans* (3;4), in some of these countries it is the most important cause of meningitis (5).

Mortality of cryptococcosis is high. Between 10 and 25% of AIDS patients die during initial treatment, and between 30 and 60% of patients succumb within 12 months (6;7). In non-AIDS patients mortality rates are even higher, between 25-30% of patients die during initial treatment.

The aim of this study was to gain insight into prevalence, clinical features and prognosis of cryptococcosis in the Netherlands. Prior to 1985, the annual incidence of cryptococcosis was 5 cases (8). We retrospectively analyzed data from 268 patients with cryptococcosis from January 1986 through December 1999. In July 1996, highly active antiretroviral therapy (HAART) for HIV-infected patients was widely introduced in the Netherlands. This allowed us to also evaluate the effect of HAART on the incidence of cryptococcosis.

PATIENTS AND METHODS

Study population and source

From January 1986 through December 1999 data from patients with cryptococcosis were retrospectively collected. Patients were identified from the registers of the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) in Amsterdam and 5 academical microbiology laboratories (University Medical Centre Utrecht, Erasmus University Medical Centre Rotterdam, Academical Hospital Nijmegen St. Radboud, Academical Medical Centre Amsterdam, University Medical Centre Vrije Universiteit Amsterdam. The NRLBM receives isolates and sera of approximately 90% of all patients with meningitis in the Netherlands (9).

Case definition

Cryptococcosis was diagnosed in cases with a positive *C. neoformans* culture and/or positive antigen detection test combined with clinical suspicion of cryptococcosis and/or the presence of cryptococci by tissue histology. In 211 of 268 patients (78%) with cryptococcosis a positive liquor culture or antigen detection test was found, these patients were diagnosed cryptococcal meningitis.

Data collection

From 111 patients with cryptococcal meningitis additional data were obtained: demographic and epidemiological data (sex, age, hospital), clinical information (signs and symptoms, date of diagnosis, relevant medical history), laboratory data (source and date of culture-positive specimens, source and date of antigen detection, clinical chemistry of CSF), radiological findings (abnormalities on chest X-ray or CT scan), underlying conditions, treatment (initial therapy, duration, changes in treatment, use of antimycotic therapy), and complications and outcome (death related to cryptococcosis, clinical cure or improvement, relapse). Patients were considered to have died from cryptococcal meningitis if they had a positive CSF culture or antigen test for *C. neoformans* at the time of death, in combination with fitting clinical symptoms. Patients were classified as cured when clinical symptoms disappeared and CSF cultures were repeatedly negative during a follow-up period of at least one year. Patients were classified as improved when clinical symptoms disappeared and

cultures of CSF were negative when last examined, but follow-up was for less than one year, or when the patient died of other causes after successful treatment of cryptococcal infection. Relapse was defined as a negative culture after treatment followed by a positive culture for *C. neoformans* in combination with fitting clinical symptoms. Positive outcome was defined as cure or improvement, negative outcome as death from cryptococcal infection or relapse. Patients were scored only once; relapses were not counted as a separate event.

Statistical analysis

Variables were compared using chi-square test and univariate analysis (Mann-Whitney U test). With potential prognostic factors from univariate analysis a multi variate analysis was performed. Differences were considered significant with a P value < 0.05.

RESULTS

Prevalence of cryptococcosis in the Netherlands

During 14 years 268 patients were diagnosed with cryptococcosis. Cases of cryptococcosis were noted throughout the Netherlands, but the majority of patients originated from the two largest cities, Amsterdam and Rotterdam (fig. 1).

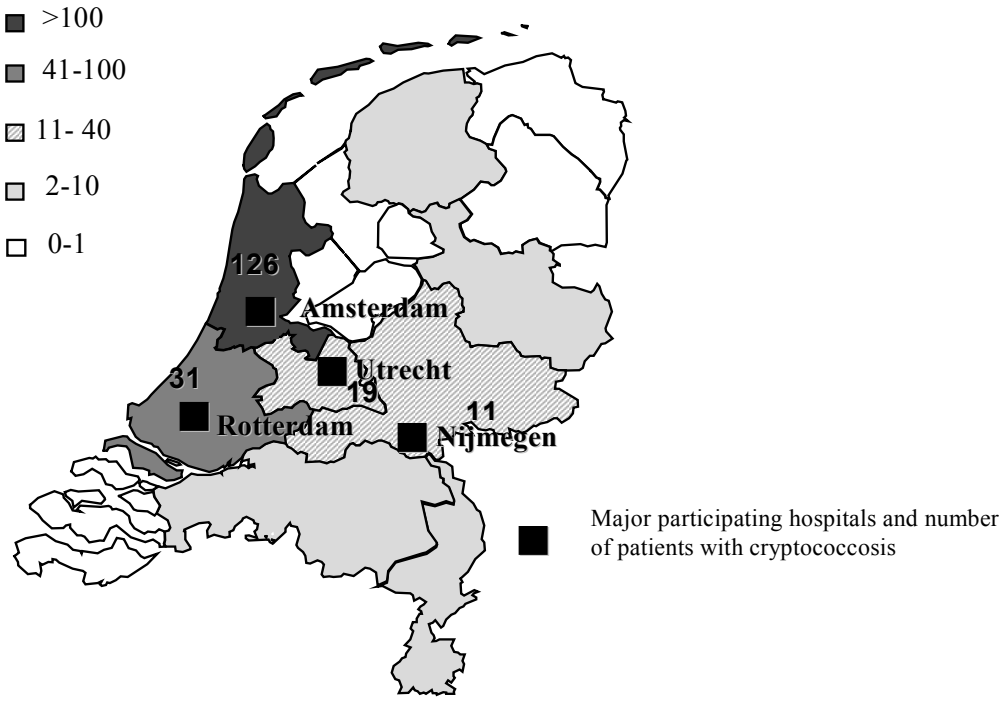

Figure 1: Geographical distribution of cryptococcosis in the Netherlands (1986-1999). The number of patients with cryptococcosis is shown in different tones of gray.

A sudden major increase in the number of patients with cryptococcosis was seen in 1987, virtually completely due to the increasing number of HIV-related cases (fig. 2). During the first 10 years covered by the survey, the mean annual incidence of cryptococcosis was 21.3 cases per year. This declined by 55% to only 9 and 10 cases per year in 1996 and 1997, respectively, and 13 cases per year in 1998 and 1999. This decline was caused by reduction of HIV-infected patients with cryptococcosis.

The number of non-HIV-infected patients with cryptococcosis remained stable throughout the 14-year period, varying from 1 to 6 cases a year (fig.2). The decline of cryptococcosis in HIV-infected patients could not be explained by a lower incidence of AIDS patients. There was however, a clear association with the introduction of HAART in the Netherlands in 1996. Most of the AIDS patients diagnosed before 1996, and newly diagnosed patients were given HAART. Most of these patients were included in the ATHENA trial, a national multi center clinical cohort study started in 1998 to evaluate the effect of HAART.

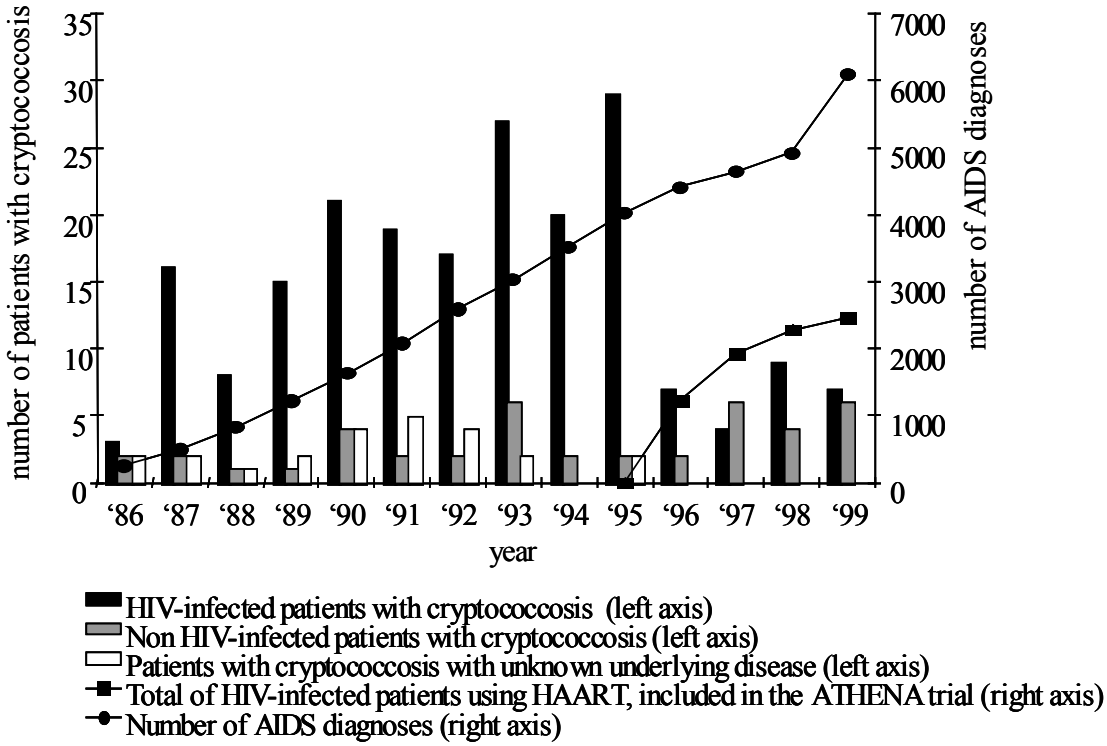

Figure 2: Number of patients with cryptococcosis, AIDS and patients on HAART during 1986-1999.

Characteristics of patients

Of 268 patients with cryptococcosis, 203 were diagnosed with HIV infection (table 1). No predisposing factors could be detected in 7 patients despite an extensive work-up. One of these patients was known to work in a bird sanctuary. Two patients were pregnant which is a known risk factor for cryptococcosis (10).

Table 1: Age, sex and predisposing factors of 268 cryptococcosis patients

Age (median)	39 (range 15-78)
Sex (male: female)	8:1
Predisposing factors	
- None	7
- Unknown	24
- HIV-infection	203
- Use of immunosuppressive drugs	14
- Hematological malignancy	16
- Cellular immune deficiency	2
- Pregnant	2

Clinical manifestations of patients with cryptococcal meningitis

Cryptococcosis is a systemic infection, commonly presenting as meningoencephalitis. In the next part of this chapter we focus on patients presenting with cryptococcal meningitis. Clinical presentation of patients with cryptococcal meningitis at the time of presentation at the hospital is counted up in table 2. The median duration of symptoms before presentation was 24.5 days (range 2-125 days), with 30% of patients having been symptomatic for more than 25 days. HIV-infected patients were diagnosed within 3 days after hospital admission whereas non-HIV-infected patients were diagnosed after 10 days ($P<0.01$). HIV-infected patients presented more often with headache than non HIV-infected patients ($P<0.05$). HIV-infected patients also tended to present more often with symptoms compatible with encephalopathy (photophobia, seizures) than those without HIV infection. Most of the HIV-infected patients had advanced disease with a median CD4+T-cell count of 30 cells/(l (range 3-240). At the time of hospital admission 23 patients (21%) used azoles (mainly Fluconazole) because of recurrent mucosal candidiasis and were apparently not properly protected.

Table 2: Clinical presentation of patients with cryptococcal meningitis at time of hospital admission according to HIV status (n=111)

Symptoms	**HIV-infected patients** (n=97)	**Non-HIV infected patients** (n=14)
Fever	83 (85%)	12 (86%)
Headache	87 (90%)	8 (57%)*
Cough	35 (31%)	2 (14%)
Nausea	47 (48%)	4 (27%)
Vomiting	40 (41%)	6 (43%)
Malaise	59 (61%)	7 (50%)
Rigidity of the neck	27 (28%)	2 (14%)
Changes in consciousness	26 (27%)	5 (36%)
Photophobia	18 (18%)	1 (7%)
Papilledema	10 (10%)	2 (14%)
Seizures	12 (12%)	0
Visual changes	14 (14%)	3 (21%)
Cranial nerve lesion	11 (11%)	0
Hemi paresis	3 (3%)	0
Duration of symptoms at first presentation (days)	21	18
Time between first presentation and diagnosis (days)	3	10†

* $P<0.05$

† $P<0.01$ (Mann Whitney U-test)

Diagnostic features of patients with cryptococcal meningitis

CSF cultures were positive for *C. neoformans* in 108 of 111 cases (97%). Blood cultures were performed in 66 patients and were positive for *C. neoformans* in 28 patients (42%) (table 3). Opening pressure from lumbar puncture (normal: <12 mmHg) was elevated in 96% of patients (median 30 mmHg, range 8-80 mmHg). The number of leucocytes in the CSF was 12 x 10^6 cells/3 per mm^3 for HIV-infected patients, 269 x 10^6 cells/3 per mm^3 for non-HIV-infected patients.

A chest X-ray was made in 95 patients with cryptococcal meningitis and revealed abnormalities in 37 (39%). These ranged from very small, delicate lesions to massive infiltrates in the lungs. Cranial computer-tomographic (CT) scanning demonstrated abnormalities (ventricular enlargement, hypodense lesions, hyperdense spot-like lesions, and cortical atrophy) in 28 out of 86 patients.

Table 3. Laboratory and radiological findings according to HIV status of patients with cryptococcal meningitis

	Total (n=111)	**HIV-infected patients** (n=97)	**Non HIV-infected patients** (n=14)
CSF (median, range)			
-Opening pressure (mmHg)	30 (8-80)	30 (8-80)	ND
-Glucose (mmol/l)	2.7 (0.1-4.8)	2.6 (0.1-4.8)	3.8 (1.0-4.8)
-Protein (g/l)	0.68 (0.15-5.7)	0.66 (0.15-5.7)	0.95 (0.3-2.13)
-Cell count (cells/3 per mm^3)	243 (0-1153)	12 (0-1153)	269 (42-350)*
-Cryptococccal antigen titer	1:328 (1:1-1:1x10^6)	1:400 (1:1-1:1x10^6)	1:64 (1:4-1:6,6x10^5)
-Pos. CSF-culture	108/111 (97%)	95/97 (98%)	13/14 (93%)
Blood			
-Cryptococcal antigen titer	1:1024 (1:2-1:2,6x10^5)	1:1034 (1:2-1:2,6x10^5)	1:256 (1:4-1:1024)
-Pos. blood culture	28/66 (42%)	25/58 (43%)	3/8 (38%)
Radiology			
Chest X-ray -Abnormal	37/95 (39%)	33/85 (39%)	4/10 (40%)
Cranial CT-scan -Abnormal	28/86 (32%)	24/76 (32%)	4/10 (40%)

* P<0.05 (Mann-Whitney-U) test
ND= not done

Therapy of patients with cryptococcal meningitis

Treatment data were available for 108/111 patients. Fifty-four patients were initially treated with intravenous amphotericin B (dose 0.3-0.7 mg/kg/day) in combination with either oral or intravenous 5-fluorocytosine (5-FC) (dose 150 mg/kg/day). Other commonly used initial therapies were intravenous amphotericin B (dose 0.5-0.7 mg/kg/day) (n=16) or oral azoles (n=23). A liposomal formulation of amphotericin B, Ambisome®, was used in 7 patients, twice in combination with fluconazole.

Cryptococcal meningitis was diagnosed post-mortem in 4 cases. Tuberculosis was suspected in one case and anti-tuberculosis treatment was started. Three cases were considered to have suffered from viral meningitis and were treated with acyclovir.

Toxic effects of initial treatment, such as thrombocytopenia and granulocytopenia for 5-FC (n=9) and nephrotoxicity for amphotericin B (n=4), resulted in changes of therapy. No clinical improvement was observed in 7 patients using fluconazole as initial monotherapy, in these cases amphotericin B was added. Symptomatic relief was achieved by repeated lumbar punctures in 3 patients with severe headache and high lumbar opening pressures. All HIV-infected patients received long-term maintenance therapy with either fluconazole (88%) (dose 150-400 mg/day) or itraconazole (12%) (dose 200-600 mg/day).

Outcome of patients with cryptococcal meningitis

Mortality of cryptococcal meningitis was 19.5% (n=19) in HIV-infected and 28.5% (n=4) in non-HIV infected patients. Seven HIV infected patients were cured, 56 patients improved clinically. Nine non-HIV infected patients improved clinically. In 5 cases of HIV infected patients and in 1 non-HIV infected patient no information about outcome was available. Ten patients, all HIV infected, had a relapse. Altered consciousness ($P<0.01$), seizures ($P<0.01$), rigidity of the neck ($P<0.05$) and a high cryptococcal antigen titer in CSF at the moment presentation, were associated with a negative outcome in univariate analysis (table 4). Initial therapy with amphotericin B and 5-FC was associated with a positive outcome as compared to other therapies ($P<0.05$). In logistic regression only altered consciousness was associated with a negative outcome ($P<0.05$).

DISCUSSION

Changes in the occurrence of cryptococcosis are mainly caused by changes in the epidemiology of AIDS in the Netherlands since 1986. From 1996 on a sharp decline in the incidence of cryptococcosis was noticed. This decline was caused by reduction of HIV-infected patients with cryptococcosis. The estimated incidence of HIV infection in the Netherlands in this same period did, however, not decrease (11). Since the introduction of HAART in 1996 a decline in the number of patients with progression from HIV infection towards AIDS has been noticed (12). This is explained by the increase of CD4+-cells, which goes with a restored immune system and a better host defense against opportunistic infections (13-16). The decline of HIV-infected patients with cryptococcosis is probably explained by the partial restoration of the immune system as a result of HAART (17).

The decline in incidence of cryptococcosis starts in the beginning of 1996, around the introduction of HAART, could possibly also be explained by the introduction of azoles, mainly fluconazole, for recurrent mucosal candidiasis (18). However, in our study 23 patients became infected with *C. neoformans* despite the use of antifungal

agents. Furthermore in the Netherlands primary prophylactic antimycotic agents against recurrent mucosal candidiasis was not recommended.

Table 4: Prognostic features for cryptococcal meningitis

	No. of patients	No. of patients who died/relapsed	*P*-value
Clinical presentation			
-Altered consciousness			<0.01*/<0.05†
Yes	30	16	
No	75	17	
-Seizures			<0.01*
Yes	12	8	
No	93	25	
-Rigidity of the neck			<0.05*
Yes	28	13	
No	77	20	
Treatment			
-Amphotericine B + 5-FC	53	11	<0.05*
-Other	50	22	
Laboratory findings			
-Antigen titer in CSF	1:256	1:1024	<0.05#

* derived by Fisher's exact test
# derived by Mann Whitney U-test
† multivariate analysis in a logistic regression model

Severe neurological symptoms (like altered consciousness) and a high antigen titer at the time of presentation are both associated with negative outcome. The severity of the clinical symptoms is probably caused by infection of cryptococci with high fungal load. As a result of an impaired immune system, cryptococci get the chance to multiply and cause severe damage to surrounding tissues. Treatment of cryptococcal meningitis for both HIV, and non-HIV-infected patients with amphotericin B in combination with 5-FC is associated with a positive outcome, as compared to the other treatment options. Next to initial treatment life long secondary prophylaxes with oral azoles was indicated for patients with HIV-infection. Very recently, there are several studies suggesting that it is safe to stop secondary prophylaxis against *C. neoformans* in patients responding to HAART (increase in CD4+ T cell count to >50-

100 cells/3 per mm^3), without relapse of their cryptococcal disease (19;20). In our study 9 patients developed cryptococcal meningitis with a CD4+ T cell count >100 cells/3 per mm^3. Stopping secondary prophylaxis in a HIV-infected patient will, beside the increase of CD4 cells, also be dependent on the clinical condition of the patient.

C. neoformans is worldwide one of the most important causes of meningitis, however in the Netherlands cryptococcosis is a rare finding. In all cases of sub-acute or chronic meningitis, and especially in patients with impaired immunity cryptococcal-associated disease belongs in the differential diagnosis. Because mortality rate of crytococcal meningitis for both HIV, and non-HIV-infected patients is high, fast recognition of clinical symptoms and awareness of risk factors for cryptococcosis remain important.

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CHAPTER 3

Quantitative analysis of phagocytosis of *Cryptococcus neoformans* by adherent phagocytic cells by fluorescence multi-well plate reader.

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ABSTRACT

Macrophages and monocytes are adherent phagocytic cells, which play an important role in host defence against the yeast-like fungus *Cryptococcus neoformans*. Before, phagocytosis by adherent phagocytes could only be measured by means of microscopy or by a radioactive assay, which both have obvious disadvantages. We have developed a new, rapid and objective method to measure phagocytosis of *C. neoformans* by adherent phagocytes (e.g. alveolar macrophages) using a fluorescence multi-well plate reader. This method allows us to discriminate accurately between adherence and internalisation of *C. neoformans* by macrophages during long-term incubation. In addition, the method was used to study the role of the mannose receptor in phagocytosis of the acapsular yeast in the absence of serum by human monocyte-derived macrophages (MDM).

INTRODUCTION

Cryptococcus neoformans is a yeast-like fungus that causes infections in immunocompromised patients, especially in those with AIDS. Cryptococcosis caused by *C. neoformans*, is worldwide the leading cause of life-threatening mycological disease of the central nervous systems (1).

In nature, the yeast cells possess minimal capsules and are therefore easily aerosolized. In this state, the yeasts are of a smaller size and can be inhaled to the level of the alveolus (2). The initial site of infection is the lung, where (alveolar) macrophages represent the first line of host defence (3). One of the first steps in host defence, therefore, is the interaction between the acapsular *C. neoformans* and alveolar macrophages. This interaction takes place in the alveolar spaces, where only low concentrations of serum components are present (3). Cross *et al.* described that ingestion of acapsular *C. neoformans* by mice macrophages in the absence of serum occurs via constitutively present mannose- and β-glucan- receptors (4).

Previously, we have described a quantitative analysis of phagocytosis of *C. neoformans* by peripheral blood mononuclear cells using flow cytometry (5). This method enables us assessment of phagocytosis by cells in suspension, but not by adherent cells. As macrophages, like monocytes, strongly tend to adhere to different surfaces, a method is needed to measure phagocytosis by adherent cells. Until now, interaction between adherent phagocytic cells and *C. neoformans* could be studied by means of microscopy, which has the major disadvantage of being cumbersome and interpretation of results is subjective, or by a radioactive assay. This last method has disadvantages for routine laboratory use concerning health hazards and costs associated with radioisotope use and disposal. Phagocytosis of *C. neoformans* by monocytes and macrophages, measured by these methods, has been described by several investigators (6;7).

We describe a new, rapid and objective method to measure phagocytosis of *C.*

neoformans by adherent monocytes and macrophages during short and long term incubation. Fluorescein isothiocyanate (FITC)-labelled *C. neoformans* were added to adherent phagocytes in a 96-well microtiter plate. At the end of the incubation time, trypan blue was used as a quencher of the non-internalized FITC-labelled cryptococci. Interestingly, the fluorescence of the internalized cryptococci was unaffected. This concept lays the foundation of our novel phagocytosis assay. The fluorescent signal of the FITC-labeled *C. neoformans* was measured by a fluorescence multi-well plate reader before and after addition of trypan blue. This enabled us to distinguish yeast cells that had been taken up by adherent phagocytes from those that were simply attached.

The new method was compared with microscopic evaluation of phagocytosis of acapsular *C. neoformans* by monocytes or monocyte derived macrophages (MDM). Finally, the method was used to study the role of the mannose receptors in phagocytosis of the acapsular yeast by human MDM in the absence of serum.

MATERIAL AND METHODS

Cryptococcus neoformans preparation and FITC labelling

An acapsular mutant of *C. neoformans* (NIH B 4131) obtained from the National Institute of Health (NIH, Bethesda, MD, USA) was used throughout this study. The yeast cells were maintained on Sabouraud dextrose agar (Merck, Darmstadt, Germany) at 4°C. After harvesting, the cryptococci were heat-killed for 30 min at 80°C, the concentration was adjusted to 5 x 10^7 cells/ml and the cells were labelled with fluorescein isothiocyanate (FITC, Sigma, St. Louis, MO, USA) at 500 µg/ml in phosphate-buffered saline (PBS) for 30 min at 22°C. The yeast cells were then washed twice and small aliquots were stored in Hanks' balanced salt solution (HBSS; pH 7.4).

Human bronchoalveolar lavage cells

Bronchoalveolar lavage (BAL) was performed in different patients for diagnostic purposes. None of the patients suffered from cryptococcosis or was suspected of having this disease. Informed consent was obtained from participants. A total of 240 ml sterile saline was instilled in the right middle lobe in 60 ml aliquots and recovered by gentle aspiration. Rates of recovery ranged from 60 to 85%. Human bronchoalveolar lavage cells (HBALC) were then centrifuged (1200 rpm) and harvested. The supernatant was then removed. If the pellet contained erythrocytes, the pellet was treated for 5 seconds with 1 ml of 1:4 diluted PBS (sterile water). The pellet contained predominantly alveolar macrophages (>95%) as was determined by a differential cell count of a sample of the pellet using a Diff-Quik (Baxter, Unterschleissheim, Germany) stain. Viability of the cells was more than 95% as checked by trypan blue staining. The cells were adjusted to the desired concentration (1 x 10^7 cells/ml).

Isolation of monocytes and MDM

Human monocytes were isolated from buffy coats, obtained from the Blood Bank Utrecht, The Netherlands. Firstly, peripheral blood mononuclear cells (PBMC) were separated from the polymorphonuclear cells (PMN) fraction by centrifugation on a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden). Then monocytes were purified from PBMC by countercurrent elutriation as described before (8). For our experiments the >80% pure monocyte cell fractions (viability >95%) were used. For preparation of MDM, monocytes were cultured in complete medium for 7 days in Teflon erlenmeyer flasks (Nalgene, Rochester, NY, USA) at a concentration of 2 x 10^6/ml. Cells were recovered from Teflon flasks by washing, and resuspended to a final concentration of 5 x 10^7 cells/ml in RPMI 1640. At this time >95% of these cells were MDM checked by Diff-Quik (Baxter) stain and anti-CD 14 PE-labelled antibody (Beckon Dickinson, Mountain View, CA, USA). Viability (>90%) was checked by trypan blue exclusion.

Determination of association of *C. neoformans* with macrophages or monocytes by multi-well plate reader

Initial experiments showed optimal results with of 5 x 10^5 adherent cells per well. Purified macrophages or monocytes (100 µl, 5 x 10^6 cells/ml) were added to a flat-bottomed 96-well microtiter plate (Costar). The plate was placed in a CO_2 incubator for 1h at 37°C.

The FITC-labelled cryptococci were preopsonized with either 10 % human serum, including complement, or 10 % rabbit serum, including specific rabbit anti-cryptococcal antibodies (K1228), or only buffer (HBSS 1% BSA) for 30 min at 37°C. The K1228 antiserum (a kind gift of Dr. J. Orendi, Leiden, The Netherlands) was raised in rabbits against a NIH 37, a thinly encapsulated cryptococcal strain. The concentration of the preopsonized cryptococci was adjusted to 5 x 10^6 cells/ml (effector to target ratio 1:1) and subsequently added to the adherent phagocytes. All experiments were performed in quadruplicate. The plates were incubated in a CO_2 incubator for 1-24 h at 37°C. After this incubation period each well was washed twice with HBSS to remove non-bound yeast cells. The green fluorescence of the FITC-labelled *C. neoformans* was measured in a fluorescence multi-well plate reader, Cytofluor II (PerSeptive Biosystems) at 485 nm, and represents the association (binding and internalization) of *C. neoformans* with the adherent phagocytes. Supernatant was removed and 100 µl of 400 µg/ml Trypan Blue (Merck) in HBSS was added for 2 min and washed away to quench the FITC-labelled *C. neoformans* that have not been ingested by the phagocytes. The plate was read again by the fluorescence multi-well plate reader to measure the cryptococci that had been taken up by the adherent phagocytes. Only cryptococci that were inside a phagocyte still bear the fluorescent label (5).

Microscopy

We compared phagocytosis between the new method using the multi-well plate reader and microscopy. Therefore, after measuring in the fluorescence multi-well plate reader, the plates were Diff-Quik (Baxter) stained. The number of macrophages or monocytes with *C. neoformans* per 100 macrophages/monocytes (phagocytosis index) was counted. To further distinguish yeast cells that had been taken up by adherent phagocytes from those that were simply attached, wells were treated with Fungiqual A, according to the manufacturer's instructions (Reinehr/Rembold, Kandern, Germany). After washing, the wells were treated with 1% Fungiqual A in PBS for 15 minutes at 37^{o}C, washed again and examined under the fluorescence microscope. Fungiqual A stains chitin in the plasma membrane of fungi and fluoresces under UV light excitation. Again, the number of macrophages or monocytes with internalized cryptococci -as determined by Fungiqual A staining- per 100 macrophages/monocytes was counted (phagocytosis-index) and compared to the result of the fluorescence multi-plate reader.

Inhibition assay

To study the role of the mannose receptors in phagocytosis of the acapsular yeast in the absence of serum, *C. neoformans* (5. 10^6 cells/ml) were added to adherent MDM (5.10^6 cells/ml) in the presence and absence of 1 mg/ml mannan (derived from *S. cerevisiae*, Sigma). The plates were incubated in a CO_2 incubator for 3 h at 37°C and phagocytosis was measured by reading the plates by a fluorescence multi-well plate reader**.**

RESULTS

Association of *C. neoformans* with elutriated monocytes.

In order to study binding and uptake of acapsular *C. neoformans* by the adherent phagocytes, we pre-opsonized the yeast cells with either 10 % normal serum or K1228 serum (including specific rabbit anti-cryptococcal antibodies), or buffer only. Subsequently cryptococci were added to the adherent monocytes for either 3 or 18 h. After 3 h of incubation, the yeast cells which were pre-opsonized with human pooled serum (HPS) showed enhanced binding and uptake by the adherent cells, as compared to the K1228 serum and buffer pre-opsonized *C. neoformans* (Fig 1A). However, incubating the adherent monocytes with *C. neoformans* for 18 h showed roughly the same fluorescence values for all conditions tested indicating that, despite the use of different opsonins for the yeast cells (either 10% HPS or 10% K1228 serum, or buffer), phagocytosis takes place to the same extent after prolonged incubation (Fig 1B).

The same experiments were repeated with human alveolar macrophages. Different opsonized cryptococci were added to adherent human alveolar macrophages for 3 and 18 h. After 3 hours of incubation, the yeast cells, which were pre-opsonized with K1228 serum and normal serum, showed enhanced binding and uptake by the adherent cells, as

compared to the buffer treated *C. neoformans*. Again, after 18 h, the differences between fluorescence values for the differently opsonized cryptococci disappeared, indicating that phagocytosis rates were the same for all different circumstances tested (data not shown).

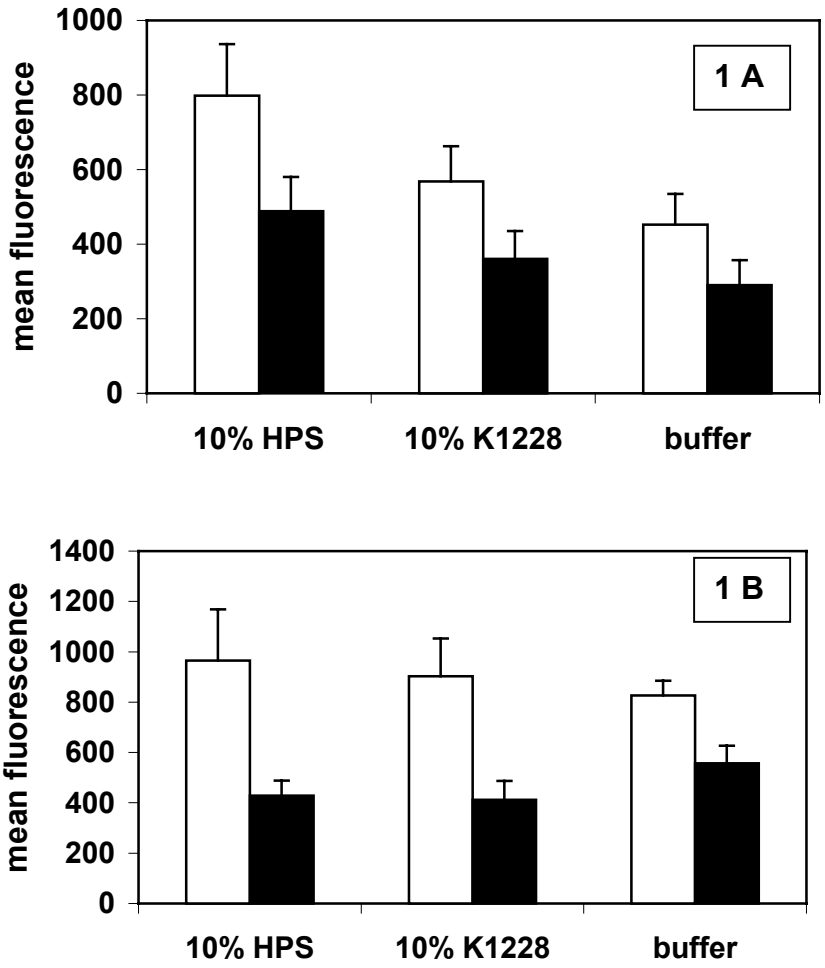

FIG 1A, 1B: Binding and uptake of FITC-labelled *C. neoformans* by adherent monocytes. Acapsular FITC-labelled *C. neoformans* (5.10^6 cells), were preopsonized with either 10 % human pooled serum, or 10% K1228 serum (including specific rabbit anti-cryptococcal antibodies), or buffer (unopsonized) for 1 h at 37ºC. Subsequently the yeasts were added to elutriated monocytes for 3 hours (A) or 18 hours (B) at an effector:target ratio of 1:1. Open bars represent binding of cryptococci, solid bars represent uptake of *C. neoformans* by monocytes. Data are presented as mean fluorescence plus SEM, and are the mean of four experiments performed in quadruplicate.

Determination of association of *C. neoformans* with monocyte derived macrophages and comparison with light microscopy.

In order to determine binding and uptake of *C. neoformans* by adherent MDM, the yeast cells were pre-opsonized as described above and added to the adherent phagocytes. After 3 h of incubation, the *C. neoformans* opsonized with 10% K1228 serum showed higher binding and uptake by the macrophages as compared to 10% normal serum opsonized

and buffer opsonized cryptococci (Fig 2A). We compared the new method with the available standard technique: microscopy. Therefore, at the end of the incubation time, half of the number of wells was measured by multi-well plate reader and half of the number of wells was treated with Fungiqual A. We compared the result of the 3 h incubation of adherent MDM with different opsonized cryptococci measured by fluorescence reader (Fig 2A) with the result of the same experiment measured by microscopy (Fig 2B). The cryptococci incubated with 10% K1228 serum showed a phagocytosis index of 50, the normal serum pre-incubated yeast cells had an index of 25. The buffer incubated *C. neoformans* had a phagocytosis index of 3 (Fig 2B). Figs 2C and D show a representative phagocytosis experiment examined by standard light microscopy.

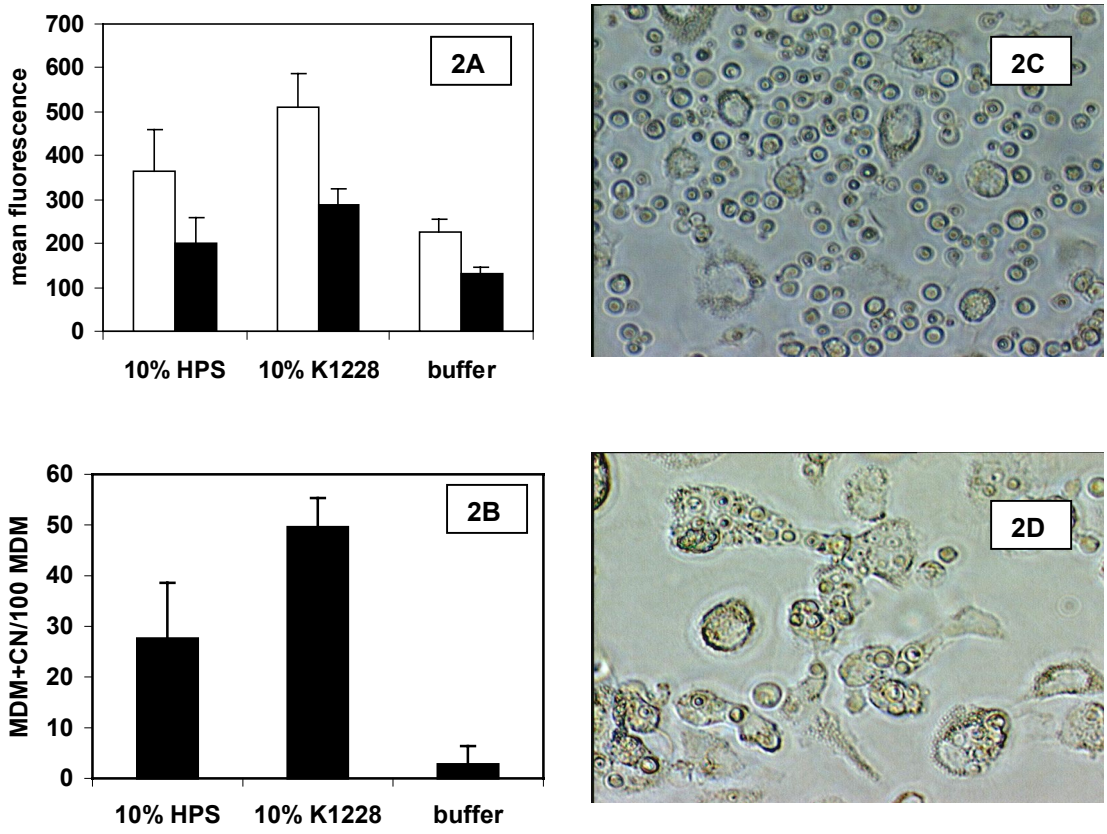

FIG 2A-D: Binding and uptake of FITC-labelled *C. neoformans* by adherent MDM; a comparison of the Fungiqual A assay (A) to the microscopical analysis (B to D). Acapsular FITC-labelled *C. neoformans* (5 x 10^6 cells), were preopsonized with either 10% human pooled serum, or 10% K1228 serum, or buffer (unopsonized) for 1 h at 37ºC. Subsequently the yeasts were added to MDM for 3 hours at an effector:target ratio of 1:1. Fig 2A: Open bars represent binding of cryptococci, solid bars represent uptake of *C. neoformans* by MDM. The plotted data were taken from a representative experiment; n=3. Fig 2B: In the same experiment the plates were treated with Fungiqual A and uptake of different opsonized *C. neoformans* by MDM after incubation of 3 hours was assessed microscopically. The number of macrophages with ingested yeast cells per 100 macrophages was counted. The plotted data are taken from a representative experiment, n=3. Fig 2C and D: Phagocytosis experiment examined by standard light microscopy. Phagocytosis of *C. neoformans* non-opsonized (C) or opsonized with 10% human pooled serum (D) by MDM (E:T 1:5) was allowed for 3 hours.

Inhibition of binding and uptake of acapsular *C. neoformans* by monocyte-derived macrophages with mannan

To study if acapsular *C. neoformans* is bound and taken up by human MDM via the mannose receptor, phagocytosis experiments were performed in the presence or absence of 1 mg/ml mannan. Binding of cryptococci by MDM in the presence of mannan was inhibited by 25%, whereas the uptake of *C. neoformans* by MDM was inhibited by 32%. However, lower doses of mannan did not show any inhibitory effect on either binding or uptake of *C. neoformans* by MDM (data not shown).

DISCUSSION

The acapsular form of *Cryptococcus neoformans* is inhaled by the respiratory tract, and can, because of its size, reach the alveolar spaces. There the yeast will meet the first cells involved in host response: the alveolar macrophages. In vitro these cells, like monocytes, tend to adhere strongly to surfaces. Therefore, to study the first line of host defence against *C. neoformans*, a system is needed to measure the interaction between cryptococci and adherent phagocytes. A new, rapid and objective method, to measure interaction between the yeast *C. neoformans* and adherent phagocytic cells is described here. A strong advantage of the described technique compared to flow cytometry is that phagocytosis of adherent cells can be measured instead of cells in suspension and, therefore, long term incubations can be studied – which is necessary when phagocytosis by non-professional e.g. endothelial cells (9). This way of studying this interaction probably resembles the *in vivo* situation more closely.

The results of the phagocytosis experiments performed with heat-killed acapsular *C. neoformans* and adherent monocytes (Fig. 1) showed that differences in opsonic conditions in combination with the phagocytosis time influence the rate of phagocytosis. After 3 h of incubation differences in phagocytosis rates of differently opsonized cryptococci could be detected. However, after 18 h of incubation, these differences disappeared. This could mean that opsonins play their role early in the phagocytosis process. We used heat-killed cryptococci since we (5) and others (10; 11) have previously shown that heat-inactivation does not affect binding nor uptake by various effector cells.

Up to now the interaction between adherent phagocytes and *C. neoformans* was measured by means of microscopy or radioactivity assays. Both methods have disadvantages. The results we obtained using this new method was very comparable with the results obtained by the routinely used microscopy (Fig 2A-D). However, this novel method is much faster, less cumbersome and less objective. The only disadvantage of the displayed technique is that the provided values are, albeit quantitative, relative instead of absolute.

To study whether the mannose receptor also plays a role in the phagocytosis of acapsular *C. neoformans* by adherent human macrophages, phagocytosis studies in the presence of

mannan were performed. The results showed that only 25% of the binding and 32% of the uptake of acapsular cryptococci by human alveolar macrophages was inhibited in the presence of mannan. In mice macrophages ingestion of *C. neoformans* was inhibited by more than 90% in the presence of the same concentration mannan (4). This possibly indicates that the expression of the mannose receptor is regulated differently in macrophages from mice to men.

In conclusion, we have developed a new and reliable method to measure phagocytosis of *C. neoformans* by different kinds of adherent phagocytes using a fluorescence multi-well plate reader. This method can be used to study the interaction between *C. neoformans* and adherent cells, during long-term incubation. In addition, the method could be used to study the interaction between other micro-organisms and adherent phagocytes. Furthermore, if a vaccine for cryptococcosis would become available, the method could for example be used to screen the opsonisation capacity of sera of vaccinated people.

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CHAPTER 4

Pulmonary Surfactant Protein A binds to *Cryptococcus neoformans* without promoting phagocytosis.

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ABSTRACT

Background: Evidence is accumulating that the alveolar collectin surfactant protein A (SP-A) plays an important role in the first line of defense against infiltrating pathogenic micro-organisms and viruses. The ability of SP-A to facilitate the binding and uptake of acapsular *Cryptococcus neoformans* by monocyte-derived macrophages, human alveolar macrophages, monocytes and polymorphonuclear leucocytes was investigated.

Materials and Methods: Binding, competition, and phagocytosis experiments were performed using a flow cytometry technique.

Results: SP-A bound to both the acapsular and the encapsulated form of *C. neoformans* in a concentration-dependent manner. SP-A showed a 3-fold better binding to the acapsular yeast, this binding was partly calcium dependent and could be inhibited by mannose (ID 50=3 mM) and glucose (ID 50=2.1 mM), but not by galactose (ID 50= 391 mM). SP-A did not function as an opsonin in phagocytosis of acapsular *C. neoformans* for any of the phagocytes studied.

Conclusion: Our results indicate that SP-A binds in a concentration-dependent manner to both encapsulated and acapsular *C. neoformans*. Despite SP-A binding to the acapsular *C. neoformans* phagocytosis by various phagocytes was not enhanced.

INTRODUCTION

Cryptococcus neoformans is a yeast-like fungus that causes infections in the immunosuppressed host. *C. neoformans* infection starts in the lung and can disseminate to other parts of the body (1). In nature, the yeast cells posses minimal capsules and are therefore easily aerosolized. In this state, the cells are of a smaller size and can be inhaled to the level of the alveolus (2). The initial step in host defense, therefore, is the interaction between the acapsular *C. neoformans* and alveolar macrophages (3).

Phagocytosis of encapsulated and acapsular *C. neoformans* by alveolar macrophages and other phagocytes has been examined by several investigators (4-6). The deposition of IgG and C3b and their degradative products on *C. neoformans* are essential for optimal phagocytosis (7). However, the concentration of these serum opsonins is low in the lungs and may, therefore, be insufficient for the effective phagocytosis of *C. neoformans* by (alveolar) macrophages and by other infiltrating leucocytes (3). Beside opsonins present in serum, other opsonic molecules present in the alveolar spaces might play a role in the first step of host defense against the yeast *C. neoformans*. We focused on two molecules present in the lung that have been described to posses opsonic functions in phagocytosis of many microorganisms; surfactant protein A (SP-A) and surfactant protein D (SP-D).

SP-A and SP-D are collagenous C-type lectins present in the surfactant layer of the lung. These two surfactant proteins, together with the plasma-defense proteins mannose-binding protein (MBP) and conglutinin, belong to the collectin superfamily, which exhibits calcium dependent mannose-specific carbohydrate binding activity (8). Previously, we showed that a member of this superfamily, the collectin human MBP, is involved in TNF-α production by monocytes stimulated by cryptococcal components (9). SP-A has been reported to act as an opsonin for a number of pathogens such as *Escherichia coli* (10), *Mycobacterium tuberculosis* (11), *Staphylococcus aureus* (12), HSV-1 (13) and the influenza A virus (14). In contrast, SP-A does not act as an opsonin for the phagocytosis of *Candida albicans* by alveolar macrophages (15), nor does it promote the phagocytosis of *Haemophilus influenzae* type B (16), *Streptococcus pneumoniae* (17), or smooth-type *E. coli* (10).

The cell wall of *Cryptococcus neoformans* is composed of a capsular polysaccharide (glucuronoxylomannan (GXM)), a minor polysaccharide (galactoxylomannan (GalXM)), and mannoproteins (MP). Each of these, respectively, consists of polymannose, polygalactose, and a protein backbone substituted with, among other saccharides, mannose (18). *C. neoformans* may therefore be recognized by the carbohydrate-binding domain of SP-A or SP-D.

Recently, Schelenz *et al.* described the binding of SP-A and SP-D to *C. neoformans* and noted that SP-D, but not SP-A, acted as an agglutinin for acapsular yeast cells (19). However, the ability of SP-A or SP-D to function as an opsonin for *C. neoformans* was not evaluated.

C. neoformans reach the alveolar spaces in an acapsular form, where resident macrophages represent the first line of defense. We examined the role of SP-A to act as an opsonin in phagocytosis of acapsular *C. neoformans* by monocyte-derived macrophages (MDM) and human alveolar macrophages (HAM). In a cryptococcosis model in CBA/J mice, Curtis *et al.* described that the composition of the leucocytic infiltrate in the lung after cryptococcocal infection consisted of monocytes/macrophages, lymphocytes ($CD4^+$ T, $CD8^+$ T) and neutrophils (20). Therefore, we examined the role of SP-A to act as an opsonin in phagocytosis of acapsular *C. neoformans* by infiltrating leucocytes and performed phagocytosis experiments with acapsular *C. neoformans* and either polymorphonuclear leucocytes (PMN) or monocytes.

Recently, Madan *et al.* showed that SP-A enhanced the binding of *A. fumigatus* conidia to PMN and monocytes (21). In order to understand more about differences in opsonic capacity of SP-A between these comparable clinically important fungi, we compared FITC-SP-A binding to acapsular *C. neoformans* and *A. fumigatus* conidia and its calcium dependency.

In this report, we discuss the binding characteristics of SP-A and SP-D to encapsulated *C. neoformans* NIH 37 and an acapsular mutant NIH B4131, using a flow cytometry technique. In addition, the role of SP-A as an opsonin in the phagocytosis of acapsular *C. neoformans* by a number of effector cells such as MDM, HAM, monocytes, and PMN was examined.

MATERIALS AND METHODS

Cryptococcus neoformans preparation and FITC labeling

Encapsulated *C. neoformans* isolate NIH 37 and an acapsular mutant NIH B 4131 were used throughout this study. The organisms were obtained from the National Institute of Health (NIH, Bethesda, MD, USA). NIH 37 is a thinly encapsulated clinical isolate (serotype A) from cerebrospinal fluid obtained from K.J. Kwon-Chung. The yeast cells were maintained on Sabouraud dextrose agar (Merck, Darmstadt, Germany) at 4º C. After harvesting, the cryptococci were heat-killed for 30 min at 80º C, the concentration was adjusted to 5 x 10^7 cells/ml and labeled with fluorescein isothiocyanate (FITC, Sigma, St. Louis, MO, USA) at 1 µg/ml in phosphate-buffered saline (PBS, pH 7.4) for 30 min at 22º C. The yeast cells were then washed and small aliquots were stored in Hanks' balanced salt solution (HBSS; pH 7.4) at -20º C until use.

Influenza A virus preparation and FITC labeling

The influenza A/DELFT/2696/89 (H3N2) virus strain was kindly provided by Dr. J.C. de Jong (Research Laboratory for Infectious Diseases, National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands). The virus was grown on the LLC monkey kidney (MK2D) cell line (LLC cells) obtained from ICN International (Irvine, UK) as described previously by Benne *et al*. (22). The virus preparation contained 8.6 x 10^7 plaque-forming units/ml as determined using the immunoplaque assay described by Harder *et al*. (23) For labeling purposes, 1 ml influenza virus was mixed with 0.1 ml 1 M sodium carbonate buffer (pH 9.6) containing 1 mg FITC/ml (Isomer I; Sigma) and incubated for 1 hour at room temperature. FITC-labeled influenza virus was layered on top of a gradient of 50-20% sucrose (wt/vol) at a total volume of 12 ml. Gradients were centrifuged at 10,000 x *g* for 5 h at 10º C. The virus fractions were then pooled and stored at -70º C until use.

Aspergillus fumigatus preparation and FITC labeling

Spores were obtained from clinical *A. fumigatus* isolates (Academic Hospital Utrecht, The Netherlands). Cultures were maintained on Sabouraud dextrose agar slants. After harvesting, the conidia were heat-killed for 30 min at 80º C, the concentration was adjusted to 5 x 10^7 cells/ml. The conidia were labeled by incubating 5 x 10^7 conidia with 0.03 mg/ml FITC (Sigma) in 1 M carbonate buffer (pH 9.0) overnight at 4º C

under mild agitation. After labeling, the conidia were washed twice with HBSS 1% BSA and stored in HBSS at -20º C until use.

Pulmonary SP-A, SP-D, FITC-SP-A, and FITC-SP-D

SP-A and SP-D preparations were a kind gift of Dr. F. van Iwaarden (Utrecht). Pulmonary SP-A was isolated by bronchoalveolar lavage fluid of patients with alveolar proteinosis as described by Haagsman *et al*. (24). The surfactant in water (1.5 mg of protein/ml) was extracted in 1-butanol (1:50, v/v) at room temperature. The surfactant/butanol mixture was centrifuged twice at 10,000 x *g* for 20 min. The precipitated protein was dried under nitrogen and washed twice in 20 mM octyl-ß-D-glucopyranoside, 100 mM NaCl, 10 mM HEPES buffer (pH 7.4) (Life Technologies, GIBCO BRL, Paisley, UK). The proteins that were insoluble in this buffer were suspended in 5 mM HEPES, pH 7.4, and dialyzed against the same buffer for 48 h. The insoluble material was removed by centrifugation at 100,000 x *g* for 30 min. The supernatant contained the purified SP-A. The purity was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). FITC labeling of SP-A was then performed according to Van Iwaarden *et al.* (13). Prior to labeling of SP-A with FITC, SP-A was dialyzed against distilled water. SP-A (3 mg) was incubated with FITC (0.3 mg) in 200 mM bicarbonate buffer, pH 8.5 (final volume, 8 ml), for 90 min at room temperature on a rotator. After incubation, the mixture was dialyzed against distilled water for 48 h at 4º C to remove the free FITC. FITC-SP-A was stored in 200 µg/ml aliquots at -70º C until use. For isolation of SP-D, rats were instilled intratracheally with silica to increase the yield (25). SP-D was isolated and purified as described and stored at -70º C in small aliquots (26). FITC labeling of SP-D was performed as described by Crouch *et al*. (25) and van Iwaarden *et al*.(26).

Carbohydrate binding domain of SP-A

As *C. neoformans* is potentially recognized by the carbohydrate-binding domain of SP-A, we evaluated the binding capacity of the carbohydrate-binding domain of SP-A used in our assays. Avidin (1µg/ml) in 0.1 M $NaHCO_3$-buffer (pH 9.6) was coated on a polystyrene 96 well microtiter plate 100 µl/well, 16 h, 4º C (Maxisorb, NUNC, Roskilde, Denmark). After coating, the microtiter plate was blocked with 2% BSA in washing solution 200 µl/well (50 mM Tris-HCL, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Tween 20, pH 7.4,) for 60 min at 22º C. Then the plate was washed 6 times in washing solution. 100 µl of mannose-polyacrylamide-biotin conjugate (Synthsome, Munich, Germany) (1 µg/ml in washing solution containing 0.1% BSA) was added to each well and incubate for 60 min at 22° C. Then the plate was washed 6 times in washing solution. SP-A samples were diluted in buffer (100 mM Tris-HCL, 5 mM $CaCl_2$, 0.05% Tween 20 containing 0.1% BSA) in the presence or absence of 10 mM EDTA and 100 µl of the sample was applied to each well. After 1 h incubation at 22°C the plate was washed 6 times and a rabbit anti-SP-A polyclonal antibody, was

applied to the plate (0.5 µg/ml, 100 µl/well, 22° C, 1h). After washing the plate, the bound antibodies were detected with horseradish peroxidase conjugated goat anti rabbit IgG (Nordic Immunol. Lab. BV, Tilburg, NL), 100 µl/well. After 1 h incubation at 22º C the plate was stained with 150 µl/well tetramethylbenzide reagent, 100 µg/ml (Merck), 1 mM H_2O_2 in 0.1 M citric acid buffer, pH 4.0). The reaction was stopped by adding 50 µl 2 M H_2SO_4 and absorption was measured at 450 nm. A concentration- and calcium-dependent binding of the SP-A was observed (ANOVA, $p<0.05$, data not shown)

Determination of FITC-SP-A and FITC-SP-D binding to *C. neoformans* and competition by various monosaccharides

Acapsular or encapsulated *C. neoformans* (10^5 organisms) were washed twice in HBSS supplemented with 1% (wt/vol) bovine serum albumin (BSA) and 1.5 mM $CaCl_2$ (HBSS-BSA-Ca) and centrifuged at 3200 rpm for 10 min at 4º C. FITC-SP-A (conc: 0-25 µg/ml) or FITC-SP-D (conc: 0-10 µg/ml) was then added to acapsular or encapsulated *C. neoformans*, mixed gently, and incubated for 1 h at 37º C. The unbound FITC-SP-A or FITC-SP-D was then washed away and the samples were diluted in PBS and analyzed by flow cytometry. In kinetics experiments performed with acapsular *C. neoformans* and FITC-SP-A incubation times varied (0-360 min.). In the competition experiments FITC-SP-A (concentration 10 µg/ml) was added to acapsular *C. neoformans* (10^5) in the presence of different concentrations (500, 5.0, 0.5 mM) of either mannose (purity 95%, Sigma) or glucose (purity 95%, Sigma) or galactose (purity 95%, Sigma). The unbound FITC-SP-A and monosaccharides were then washed away and the samples were analyzed by flow cytometry.

Isolation monocytes and MDM

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats, obtained from the Blood Bank Utrecht, The Netherlands. PBMC were isolated by centrifugation on a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden) as described by Böyum (27). The mononuclear cell layer was then washed and resuspended in RPMI 1640 (Gibco BRL, Gaithersburg, MD, USA) medium supplemented with 5 mM Hepes, 19 mM sodium bicarbonate, 10 µg/ml gentamicin, and 10% heat-inactivated human AB serum at a final concentration of 5 x 10^7/ml. Monocyte clumping was induced by rotating at 8 rpm for 1 h at 4° C. After a 30 min incubation on ice, the resulting pellet was gently dispersed. Cells were then layered onto 4 ml of ice-cold fetal bovine serum (FBS, Gibco) and monocytes were separated from lymphocytes by sedimentation for 5-10 min at 4º C. Monocytes were washed twice in complete medium and seeded into fibronectin coated tissue culture flasks. The next day adherent cells were washed with PBS (37° C), trypsinized, and again washed. Viability (>90%) was checked by trypan blue (Merck) exclusion (live cells will not stain inside). Purity of the monocytes was always > 80% checked by microscopic

evaluation of the isolated cells stained by Diff-Quik (Baxter, Unterschleissheim, Germany). For preparation of MDM, monocytes were cultured in complete medium for 7 days in hydrophobic teflon bags (Fluoroplast Ned. BV Raamsdonksveer, The Netherlands) at a concentration of 2 x 10^6/ml. Cells were recovered from teflon bags by washing, and resuspended to a final concentration of 5 x 10^7 cells/ml in RPMI 1640. At this time >95% of these cells were MDM checked by Diff-Quik (Baxter) stain and anti-CD 14 PE-labeled antibody (Beckon Dickinson, Mountain View, CA, USA). Viability (>90%) was checked by trypan blue exclusion.

Human bronchoalveolar lavage cells

Bronchoalveolar lavage (BAL) was performed in different patients for diagnostic purposes. Informed consent was obtained from participants. A total of 240 ml sterile saline was instilled in the right middle lobe in 60 ml aliquots and recovered by gentle aspiration. Rates of recovery ranged from 60 to 85%. Human bronchoalveolar lavage cells (HBALC) were then centrifuged (1200 rpm). The pellet was treated with 1 ml (1 mg/ml) DTT (DL-Dithriothreitol, Cleland's reagent; Sigma, D-0632) in PBS. After 1 minute at room temperature 2 ml RPMI was added and the DTT was then washed away by centrifuging the cells for 10 min at 1200 rpm. The supernatant was then removed. If the pellet contained erythrocytes, the pellet was treated for 5 seconds with 1 ml of 1:4, PBS: sterile water. The pellet contained predominantly alveolar macrophages (>95%) as was determined by a differential cell count of a sample of the pellet using a Diff-Quik stain. Viability of the cells was more than 95% as checked by trypan blue staining. The cells were adjusted to the desired concentration (1 x 10^7 cells/ml).

Isolation of human PMN

Heparinized venous blood, obtained from our own mini blood donor service, was diluted in PBS. The PMN were then separated by Ficoll (Pharmacia)-Histopaque (Sigma) density gradient centrifugation. After centrifugation (20 min at 1390 rpm, RT), the neutrophil fraction was collected and washed with RPMI with 0.05% human serum albumin (HSA). The remaining erythrocytes were lysed for 45 s with sterile water, after which concentrated PBS was added to reestablish an isotonic condition. After washing, cells were counted, resuspended in RPMI at 1 x 10^7 neutrophils/ml and checked for viability and purity. The viability always exceeded 95% using trypan blue exclusion. Purity was checked by Diff-Quik stain (Baxter) cytospin slides and was always >98%.

Rat bronchoalveolar lavage cells

Rat bronchoalveolar lavage cells (RBALC) were isolated as described previously by van Iwaarden *et al* (28). Rats (male, specific pathogen-free Wistar rats) were injected intra peritoneally with a lethal dose of Nembutal. Rat lungs were lavaged via the

trachea with sterile HBSS (8 portions of 10 ml/rat). The lavage fluid was centrifuged (800 rpm, 15 min 4º C). If the pellet contained erythrocytes, the pellet was treated for 5 seconds with 1 ml of 1:4, PBS: sterile water. The pellet contained predominantly alveolar macrophages (>95%) as was determined by a differential cell count of a sample of the pellet using a Diff-Quik stain. Viability of the cells was over 95% as checked by trypan blue exclusion. The macrophages were resuspended in HBSS 1% BSA and diluted to a concentration of 1 x 10^7 cells/ml.

Determination of the association between acapsular *C. neoformans* and phagocytes by flow cytometry

For use in the phagocytosis assay, FITC-labeled acapsular *C. neoformans* were pre-opsonized with either 10% HPS (positive control), buffer (HBSS 1% BSA) (negative control), or SP-A in various concentrations (1-25 µg/ml) (1 h, 37º C), and then washed once. Opsonized acapsular *C. neoformans* (100 µl; 10^7 cells/ml) were then incubated with 100 µl phagocytes, (10^7 cells/ml MDM, HAM, monocytes, or PMN,) for 2 h at 37º C. Experiments in our laboratory showed that increasing phagocytosis time (0, 15, 30 60 and 120 min) resulted in enhanced binding and uptake of cryptococci, reaching a maximum after 1 h (6). In subsequent experiments, a phagocytosis time of 2 hours was used in order to ensure optimal phagocytosis (6). After washing, half the suspension was used to determine uptake by quenching the fluorescence of the adhered yeasts (see below). Acapsular *C. neoformans* and most of the phagocytes are of the same size, which makes it difficult to distinguish between free FITC-labeled *C. neoformans* and phagocyte-associated *C. neoformans* in the flow cytometer.

In order to distinguish between phagocytes and acapsular *C. neoformans* the different phagocytes were labeled specifically using phycoerythrin (PE)-labeled monoclonal antibodies (Mab) against specific surface proteins. The following Mab were used: anti-CD 14 PE-labeled monoclonal antibody (Beckon Dickinson) for monocytes and MDM; anti-CD 71 PE-labeled monoclonal antibody (Caltag Laboratories, Burlingame, CA, USA) for human alveolar macrophages, and anti-CD 45 PE-labeled monoclonal antibody (Caltag Laboratories) for rat alveolar macrophages. Labeling was performed as follows: the acapsular *C. neoformans* phagocyte suspension was centrifuged and the supernatant recovered. Then 5 µl Mab was added to the pellet, mixed gently, and incubated for 30 min at 4º C on ice. The pellet was then resuspended in PBS and washed once. The association of FITC-labeled yeast cells (FL1) to antibody labeled phagocytes (FL2) was subsequently analyzed with a FACScan flow cytometer (Beckon Dickinson) with computer-assisted evaluation of data (LYSYS 2). PMN could be distinguished from cryptococci in the FACScan on the basis of their scatter plots. Data were expressed as the percentage of FITC-positive cells (6).

Determination of uptake of acapsular *C. neoformans* by flow cytometry

Uptake of acapsular *C. neoformans* by the different phagocytes was determined as previously described by Chaka *et al*. (6). In order to differentiate between extracellular adhering acapsular *C. neoformans* from those that had been taken up, we used trypan blue as a quenching agent for the FITC labeled acapsular *C. neoformans*. An equal volume of trypan blue (Merck) was added to each test suspension and incubated for 10 min. Optimal quenching of acapsular *C. neoformans* (>95%) was achieved at 200 µg/ml. The green fluorescence of acapsular *C. neoformans* that have been taken up by phagocytes will not be removed by quenching as trypan blue is excluded from monocytes. After 10 min the unbound trypan blue was removed by centrifugation. The supernatant was discarded and the pellet was resuspended in 200 µl PBS. These 200 µl were used to measure in the flow cytometer. Uptake was therefore determined as the difference in the percentage of phagocytes associated with FITC labeled acapsular *C. neoformans* (FL1) before and after quenching.

Comparison of FITC-SP-A binding to acapsular *C. neoformans* and conidia of *A. fumigatus*

Acapsular *C. neoformans* and conidia of *A. fumigatus* were washed twice in HBSS-BSA-Ca buffer and centrifuged at 3200 rpm for 10 min at 4º C. FITC-SP-A (conc: 0-25 µg/ml) was then added to acapsular *C. neoformans* (10^5) or conidia of *A. fumigatus* (10^5) in the presence or absence of 5 mM EDTA, mixed gently, and incubated for 30 min at 37º C. The unbound FITC-SP-A was then washed away and the samples were diluted in PBS and analyzed by flow cytometry.

Serum

Sera from 30 healthy donors were collected, pooled, and stored in small aliqouts at -70º C (human pooled serum, HPS).

Statistics

Student's T-test and ANOVA were used for statistical evaluation. A $p<0.05$ was considered significant.

RESULTS

Characteristics of binding isolated pulmonary SP-A and SP-D to acapsular and encapsulated *C. neoformans*

Different concentrations of FITC-SP-A were incubated with acapsular (NIH B 3141) and encapsulated (NIH 37) *C. neoformans* for one hour, in a calcium ion-containing buffer, and the binding was detected by flow cytometry. A dose-response curve was observed when the acapsular mutant was incubated with various concentrations of FITC-SP-A (Fig. 1A). FITC-SP-A also interacted with encapsulated *C. neoformans* in

a dose dependant manner, but this binding was about 3- fold less as compared to the acapsular mutant (Fig. 1A). FITC-SP-D binding to acapsular *C. neoformans* was also concentration dependent (ANOVA $p<0.05$), but about 10-fold lower than FITC-SP-A binding to acapsular cryptococci (Fig. 1B). FITC- SP-D did not bind (ANOVA $p>0.05$) to encapsulated *C. neoformans* (Fig. 1B).

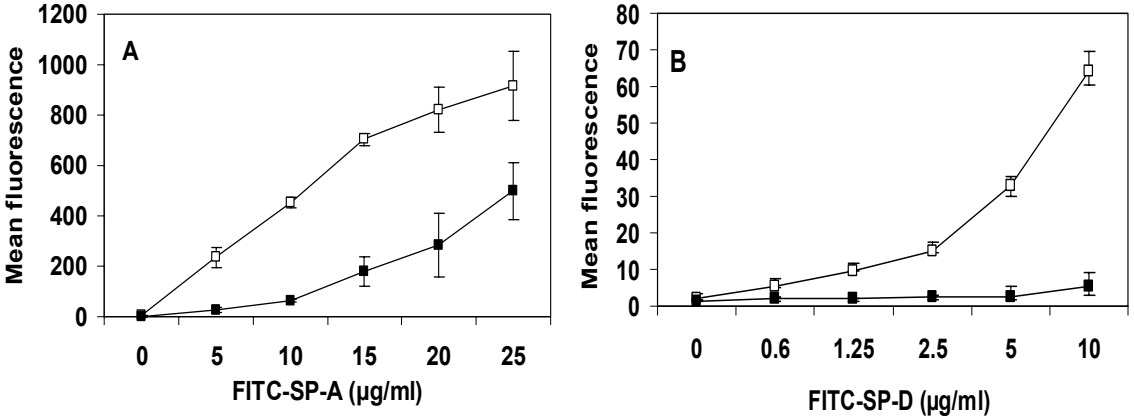

Figure 1
Concentration-dependent binding of FITC-SP-A **(A)** and FITC-SP-D **(B)** to acapsular (open squares) and encapsulated (solid squares) *C. neoformans*. *C. neoformans* (10^5 cells) were incubated with various concentrations of FITC-SP-A (0-25 µg/ml) or FITC-SP-D (0-10 µg/ml) for 1 h and analyzed by flow cytometry. Data are presented as mean fluorescence plus standard deviation, and are the mean in three experiments performed in duplicate.

Because SP-A and SP-D have been described as proteins which play a role in the first line of defense against infiltrating micro-organisms and the fact that *C. neoformans* is entering the body in an acapsular form, we choose the acapsular *C. neoformans* for subsequent experiments. In addition we choose to investigate the role of SP-A involving this first line of defense against *C. neoformans* because FITC-SP-A showed better binding to acapsular cryptococci than FITC-SP-D (Fig 1).

Next, experiments were performed to study the kinetics of binding of SP-A to the acapsular cryptococcus. FITC-SP-A (10 µg/ml) was incubated with the cryptococci for different time points (0, 7.5, 15, 30, 60, 120, 180, 240, and 360 min.). Figure 2 shows that practically no binding of FITC-SP-A was observed after 7.5 min incubation, whereas after 1 hour >95% of the FITC-SP-A had bound. Longer incubation did not enhance FITC-SP-A binding to the acapsular cryptococcus. For subsequent phagocytosis experiments we choose 1 h pre-incubation time to ensure optimal binding of SP-A to the acapsular *C. neoformans*.

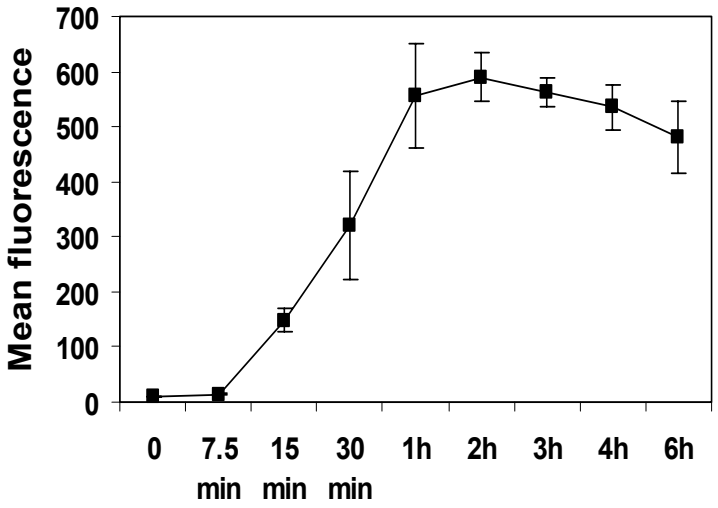

Figure 2
Acapsular *C. neoformans* (10^5 cells) were incubated with 10 µg/ml FITC-SP-A for 0 min, 7.5 min, 15 min, 30 min, 1h, 2h, 3h, 4h, and 6h and analyzed by flow cytometry. Data are presented as mean fluorescence plus standard deviations, each time point was performed in duplicate.

Carbohydrate binding domain of SP-A

Competition studies were performed to confirm that SP-A bound via the carbohydrate-binding domain to acapsular *C. neoformans*. SP-A has previously been reported to have affinity for glucose and mannose, but not for galactose residues (29).

Therefore, these three monosaccharides were used in the competition assay. For 50% inhibition (ID 50) of the FITC-SP-A binding to acapsular *C. neoformans*, 3.0 mM mannose and 2.1 mM glucose was required, whereas about a 100-fold higher concentration of galactose was needed (393 mM) (Fig 3). These data are consistent with the literature, and are suggesting that SP-A can bind to acapsular *C. neoformans* by its carbohydrate-binding domain.

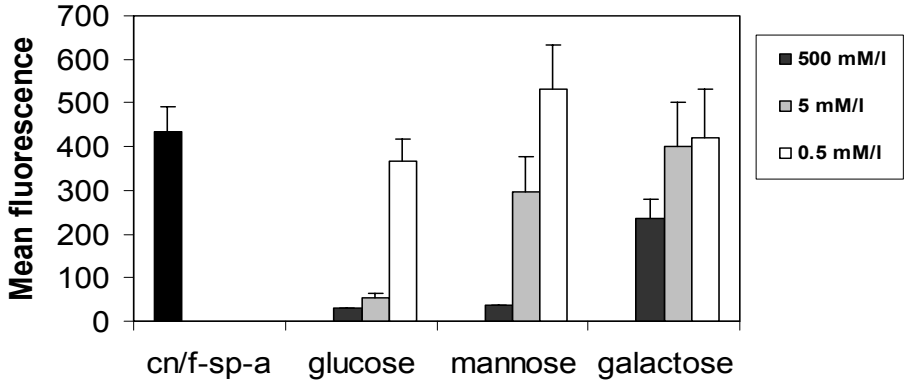

Figure 3
Competition of FITC-SP-A binding by various monosaccharides to acapsular cryptococci. FITC-SP-A (10 µg/ml) was incubated in the presence of different concentrations (500, 5.0 and 0.5 mM/l) of either mannose or glucose or galactose with 10^5 *C. neoformans* for 1 h and analyzed by flow cytometry. Data are presented as mean fluorescence plus standard deviations; each sample was performed in duplicate.

Opsonic activities of SP-A in phagocytosis of acapsular *C. neoformans* by macrophages

Acapsular yeast cells incubated with 10% HPS (including complement and IgG) were used as a positive control and acapsular yeast cells incubated with buffer were used as a negative control. Acapsular *C. neoformans* were also incubated with 20 µg/ml SP-A for 1 h (pre-opsonization) followed by incubation with MDM for 2 h. The serum-opsonized acapsular *C. neoformans* showed a significantly higher binding ($p<0.05$) and uptake ($p<0.05$) than the buffer-pre-opsonized acapsular *C. neoformans*. In contrast, SP-A pre-opsonisation of acapsular *C. neoformans* did not enhance binding and uptake by MDM when compared to the buffer pre-opsonized control (Fig. 4A).

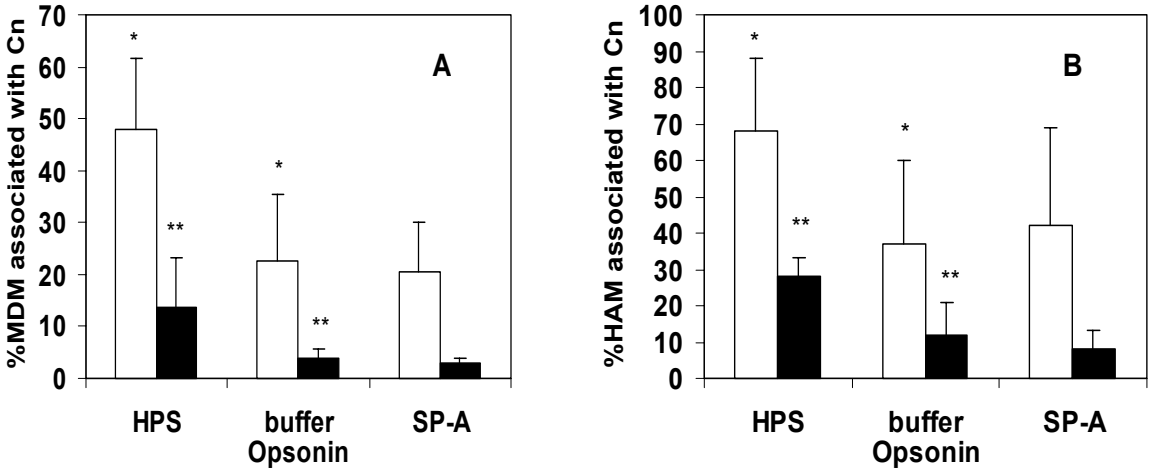

Figure 4
Binding and uptake of FITC-labeled acapsular *C. neoformans* by MDM (A), and human alveolar macrophages (HAM) (B). Acapsular FITC-*C. neoformans* (10^6 cells) were pre-opsonized with 10% HPS, buffer (unopsonized) or 20 µg/ml SP-A for 1 h at 37° C, washed once, and added to 10^6 MDM (Fig 4A) or 10^6 HAM (Fig 4B) for 2 h. After washing, the pellets were incubated with PE-labeled monoclonal antibody for 30 min at 4° C. After washing the pellets were resuspended in PBS and analyzed. Open bars represent binding of acapsular *C. neoformans* to phagocytes; solid bars represent uptake of acapsular *C. neoformans* by phagocytes. Data are the mean of duplicate incubations and standard deviations in four experiments.
4A: Differences in binding (*$p<0.05$) and uptake (**$p<0.05$) between HPS and buffer pre-opsonized cryptococci by MDM are statistically significant. Differences in binding and uptake between buffer and SP-A pre-opsonized cryptococci by MDM is not statistically significant.
4B: Differences in binding (*$p<0.05$) and uptake (**$p<0.05$) between HPS and buffer pre-opsonized cryptococci by HAM are statistically significant. Differences in binding and uptake between buffer and SP-A pre-opsonized cryptococci by HAM is not statistically significant.

Pre-incubating the acapsular *C. neoformans* with 10% serum together with 20 µg/ml SP-A showed the same percentages binding and uptake by the MDM as cryptococci that were pre-incubated with 10% serum only (n=2, data not shown). When different concentrations of SP-A were tested (0.5-50 µg/ml) or different effector-to-target cell ratios (1:5, 1:10) were used, identical results were observed (data not shown). The phagocytosis experiments were repeated with human alveolar macrophages.

Acapsular *C. neoformans* pre-incubated with 10% serum showed 65% binding and around 25% uptake by HAM. Both the non-opsonized negative control and the SP-A pre-opsonized cryptococci showed similar percentages of binding of around 30%, and uptake of around 10% (Fig 4B). This means that SP-A did not enhance phagocytosis of acapsular *C. neoformans* by either MDM or human alveolar macrophages.

Opsonic activities of SP-A in phagocytosis of *C. neoformans* by monocytes and PMN

We also tested the role of SP-A in the phagocytosis of acapsular *C. neoformans* by infiltrating leucocytes, monocytes and PMN. Monocytes showed 79% binding and 57% uptake of acapsular cryptococci that were pre-opsonized with 10% serum. Twenty five percent of the monocytes bound acapsular *C. neoformans* that were pre-opsonized with buffer, 23% of the monocytes bound acapsular *C. neoformans* that were pre-opsonized with SP-A. Uptake by monocytes of acapsular *C. neoformans* was also not enhanced by pre-incubating the yeasts with SP-A compared to the buffer control (Fig 5A).

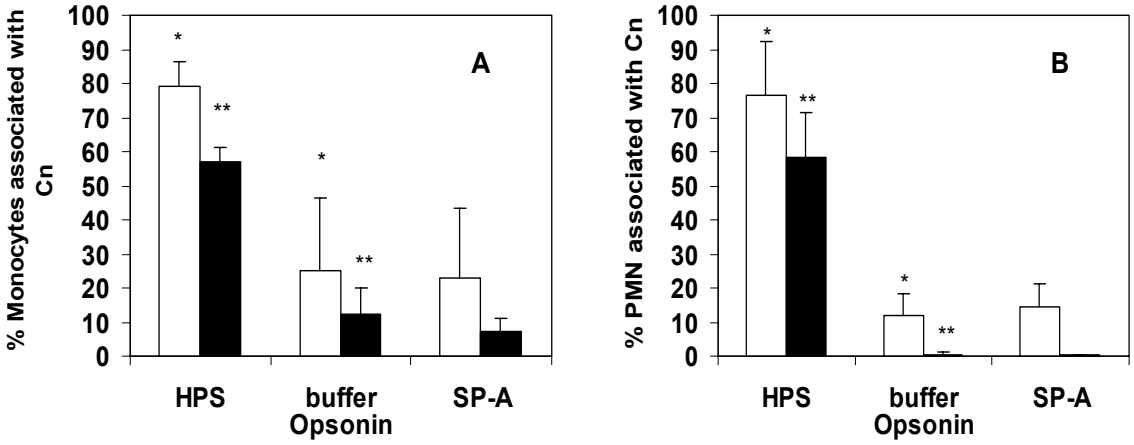

Figure 5
Binding and uptake of FITC-labeled acapsular *C. neoformans* by monocytes (A) and PMN (B). Acapsular FITC-*C. neoformans* (10^6 cells) were pre-opsonized with 10% HPS, unopsonized (buffer), or 20 µg/ml SP-A, for 1 h at 37° C, washed once, and added to 10^6 monocytes (Fig 5A) or 10^6 PMN (Fig 5B). Open bars represent binding of acapsular *C. neoformans* to phagocytes; solid bars represent uptake of acapsular *C. neoformans* by phagocytes. Data are the mean of duplicate incubations in four experiments.
5A: Differences in binding (*$p<0.05$) and uptake (**$p<0.05$) between HPS and buffer pre-opsonized cryptococci by monocytes are statistically significant. Differences in binding and uptake between buffer and SP-A pre-opsonized cryptococci by monocytes is not statistically significant.
5B: Differences in binding (*$p<0.05$) and uptake (**$p<0.05$) between HPS and buffer pre-opsonized cryptococci by PMN are statistically significant. Differences in binding and uptake between buffer and SP-A pre-opsonized cryptococci by PMN is not statistically significant.

PMN showed 78% binding and 58% uptake of acapsular cryptococci pre-opsonized with serum. The cryptococci that were pre-opsonized with buffer only showed 12% binding, the ones pre-opsonized with SP-A showed 14% binding. This difference is

not significant (p>0.05). Uptake by PMN of acapsular *C. neoformans* was not enhanced by pre-incubating the yeasts with SP-A compared to the buffer control (Fig 5B).

Opsonic function of SP-A in other organisms.

Since SP-A is an opsonin for influenza A virus phagocytosis by rat alveolar macrophages (14) FITC-labeled influenza A virus was included as an extra positive control to confirm the biological activity of the SP-A preparations used during this study. In this set of experiments, influenza A virus was pre-opsonized with either buffer, or 20 µg/ml SP-A before the rat cells were added. SP-A significantly enhanced phagocytosis (binding and uptake) of influenza A virus when compared to the buffer control. Rat alveolar macrophages (RAM) showed 11.6+/-1.8% binding of influenza A virus pre-opsonized with buffer, and 19.0+/-3.4% binding of SP-A pre-opsonized influenza A virus (p<0.05). RAM showed 7.15+/-1.2% uptake of buffer pre-opsonized influenza A virus, and 15.05+/-0.77% binding of SP-A pre-opsonized influenza A virus (n=3, p<0.05) (data not shown). Recently, Madan *et al.* showed that SP-A enhanced the binding of *A. fumigatus* conidia to PMN and monocytes (21). In contrast to *C. neoformans*, SP-A enhanced the binding of *A. fumigatus* to monocytes by 42% when compared to non-opsonized control (n=4, p< 0.05) (data not shown). Experiments were conducted to understand more about the observed differences in opsonic capacity of SP-A between the two fungi. We investigated the binding of FITC-SP-A to acapsular *C. neoformans* and *A. fumigatus* conidia. FITC-SP-A binding to *A. fumigatus* conidia was at least twofold higher than the binding to *C. neoformans*, p<0.05 (Fig. 6).

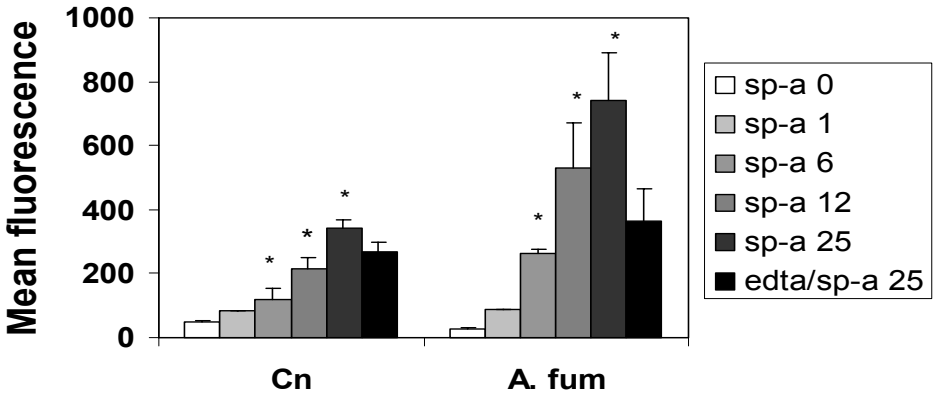

Figure 6 Acapsular *C. neoformans* (10^5 cells) and conidia of *A. fumigatus* (10^5 cells) were incubated with different concentrations of FITC-SP-A (0-25 µg/ml) for 30 min and analyzed by flow cytometry. Gray bars represent binding of different concentrations of FITC-SP-A to acapsular *C. neoformans* or *A. fumigatus* in the presence of 1.5 mM calcium buffer; black bars represent binding of 25 µg/ml FITC SP-A to acapsular *C. neoformans* or *A. fumigatus* in the presence of 5 mM EDTA buffer. Data are presented as mean fluorescence and are the mean of duplicate incubations and standard deviation in three experiments. Differences in mean fluorescence values between acapsular *C. neoformans* and conidia of *A. fumigatus* are significant (*p<0.05) after incubation of 6, 12.5, and 25 µg/ml FITC SP-A in the presence of calcium buffer.

FITC-SP-A binding to both fungi was calcium dependent, but a difference was observed. EDTA inhibited FITC-SP-A binding to *A. fumigatus* by 51%, whereas the binding to *C. neoformans* was only reduced by 21% (Fig. 6). However, no enhanced uptake of *A. fumigatus* conidia by either cell type tested (HAM, monocytes or PMN) by SP-A was seen as compared to the negative buffer control. (n=3, data not shown).

DISCUSSION

SP-A is one of the hydrophilic proteins present in the surfactant layer of the lung. It plays a crucial role in the dynamics and metabolism of the extracellular surfactant (30). Beside these functions, SP-A has also been described to act as an opsonin for a number of pathogens (10;11;13;17). SP-A concentration in BALs from healthy individuals is estimated 1-2 µg/ml but these levels are probably a 10-100 times dilution of the concentration present in the surfactant layer itself. SP-A levels in HIV-infected pneumonia patients are found to be significantly elevated with a mean of 5.23 µg/ml (31). Around 80% of patients infected with *C. neoformans* are infected with HIV (32). Therefore, we were interested in the role of SP-A in the host defense against *C. neoformans.*

FITC-SP-A bound in a concentration-dependent manner to both acapsular and encapsulated *C. neoformans.* FITC-SP-D also bound in a concentration-dependent manner to the acapsular *C.* neoformans. However, this binding was about 10-fold lower as compared with FITC-SP-A binding. FITC-SP-D did not bind to encapsulated *C. neoformans*. Therefore, subsequent experiments were performed with SP-A. In the present study, we investigated the hypothesis that SP-A enhanced the phagocytosis of acapsular *C. neoformans* by different phagocytes. This is relevant because *C. neoformans* enters the human lung in an acapsular form, before it synthesizes its capsule and disseminates throughout the body (1).

The FITC-SP-A binding to acapsular *C. neoformans* could be inhibited by glucose and mannose, but not by galactose. This means that SP-A binding to acapsular *C. neoformans* can occur via its carbohydrate-binding region. Despite SP-A binding to acapsular *C. neoformans*, SP-A did not enhance the phagocytosis of acapsular *C. neoformans* in experiments conducted with different types of effector cells. The SP-A preparations used in this study, were biologically functional. SP-A did enhance the binding and uptake of influenza A virus by rat alveolar macrophages and the binding of *A. fumigatus* conidia to monocytes. Therefore, we conclude that SP-A does not promote the phagocytosis of acapsular *C. neoformans* by phagocytes. This is in contrast to a number of bacterial and viral pathogens, such as *E. coli* (10), *M. tuberculosis* (11), *S. aureus* (17), HSV 1 (13) and influenza A virus (14) but is in agreement with the results found with the yeast *Candida albicans* (15).

Van Iwaarden *et al.* described that pre-incubation of rat alveolar macrophages with surfactant increased the phagocytosis of rat-serum opsonized *S. aureus* by 70% compared to the control macrophages. This was probably due to an upregulation of the involved receptors (28). Pre-incubation or opsonization of *S. aureus* with surfactant did not result in enhanced phagocytosis by the alveolar macrophages. We also performed phagocytosis experiments in which the phagocytes were pre-incubated with SP-A, but again no enhanced opsonic function for the SP-A could be detected (data not shown).

Schelenz *et al.* described that both SP-A and SP-D bind to *Cryptococcus neoformans,* but only SP-D induces agglutination of acapsular yeast cells (19). In addition, they found that SP-D consistently resulted in a 10- to 17-fold higher fluorescence intensity than SP-A. We found only low binding of FITC-SP-D to encapsulated and acapsular *C. neoformans*. However, as stated by the authors themselves, it is not clear in their method, whether this higher fluorescence intensity reflects a greater affinity of SP-D for the yeast cell wall since different anti-collectin antisera were used for detection. In our method, we could directly compare FITC-SP-A and FITC-SP-D binding to the cryptococcus. Since a higher FITC-SP-A binding to *C. neoformans* was observed in our initial experiments compared to FITC-SP-D, the phagocytosis experiments were only performed with SP-A.

It is not clear whether SP-A can only bind with its carbohydrate-binding region to acapsular *C. neoformans*. SP-A is a member of the lectin family, characterized by a carbohydrate recognition domain, which binds ligands in a calcium dependent manner, and a collagen tail involved in their biological function (8). Comparing the binding characteristics of FITC-SP-A to *A. fumigatus* conidia and acapsular *C. neoformans* showed that FITC-SP-A bound about twofold better to conidia than to acapsular *C. neoformans*, and that the FITC-SP-A binding to conidia was more calcium dependent. This could mean that beside SP-A binding to acapsular *C. neoformans* via its carbohydrate-binding region, another region of the molecule may be involved, that does not posses calcium specific binding. However, despite enhanced binding of SP-A to conidia of *A. fumigatus*, no enhanced uptake could be found by a number of phagocytes tested. There are, beside conidia of *A. fumigatus*, to our knowledge, no reports in literature that describe the opsonic function of SP-A in phagocytosis of a fungus.

In conclusion, our results demonstrate that SP-A can bind to *C. neoformans* but it does not function as an opsonin for the acapsular yeast's phagocytosis. Possibly, escaping SP-A mediated phagocytosis makes it more easily for *C. neoformans* to grow in the alveolar spaces, before disseminating through the rest of the body.

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CHAPTER 5

Cryptococcus neoformans and its capsular components induce similar cytokine profiles in human peripheral blood mononuclear cells.

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SUMMARY

The capsule of the yeast *Cryptococcus neoformans* consists of glucuronoxylomannan (GXM), and two minor carbohydrate antigens, galactoxylomannan (GalXM), and mannoprotein (MP). Although the structure of these cryptococcal components is biochemically different, three strains of *C. neoformans* and its purified capsular components induced a similar cytokine pattern in peripheral blood mononuclear cells (PBMC). High levels of TNF-α and substantial levels of IFN-γ were present after 3 hours of stimulation and decreased at 18 hours. In contrast, appreciable levels of IL-10 were detected at 18 hours, whereas only low levels were found at 3 hours. IL-1 β, IL-6 and IL-8 levels were high throughout the experiment. Neither whole cryptococci nor its capsule components induced an exclusive type 1 cytokine profile (associated with cellular immune functions) or a type 2 cytokine profile (associated with enhanced antibody production from B cells). Although a more type 1 like cytokine pattern was observed at the beginning of stimulation, the pattern shifted toward a more type 2 pattern at 18 hours. The time kinetics of cytokine induction by strains of *C. neoformans* and cryptococcal components might therefore provide a time-window that facilitates induction of cellular immunity.

INTRODUCTION

Cryptococcus neoformans is an encapsulated yeast-like fungus, which can cause a systemic infection, mainly in patients with impaired cell mediated immunity, especially those with AIDS (1). The capsule of *C. neoformans* is composed of a capsular polysaccharide, glucuronoxylomannan (GXM), and two minor carbohydrate antigens, galactoxylomannan (GalXM), and mannoproteins (MP) (2). These capsular components of *C. neoformans* have been shown to induce or modulate immune responses (3;4). While GXM is generally thought to be antiphagocytic and a poor immunogen, cryptococcal mannoprotein is immunogenic in rabbits and is the antigen largely responsible for the observed DTH (5). In addition, PBMC of healthy volunteers proliferate when stimulated by cryptococcal MP suggesting that MP may be one of the molecules to which cellular immunity is directed (6). Levitz *et al.* showed that *C. neoformans* with small sized capsules induce TNF-α in whole blood (7). We have also reported that cryptococcal components induce TNF-α in PBMC (8). The presence of these antigens may thus be important in eliciting protective cell mediated immune responses, a major defense mechanisms against *C. neoformans*. Induction of an adequate cellular immunity requires the presence of a type 1 cytokine profile e.g. IFN-γ, IL-2 and IL-12 (9). Type 2 cytokines (e.g. IL-6, IL-10) enhance antibody production from B cells, and for *C. neoformans* the supportive evidence for the role of natural antibody immunity has remained uncertain despite several decades of study. In the present study we investigated whether cryptococci and its isolated capsular components stimulate PBMC to secrete a cytokine profile which

facilitates an adequate cellular immunity in normal healthy subjects.

MATERIALS AND METHODS

Strains

Encapsulated strain *C. neoformans* NIH 37 serotype A was obtained from the National Institute of Health (NIH, Bethesda, Md) and clinical strains W11 and W22 were acquired from Parirenyatwa Hospital, Zimbabwe. The different strains were maintained on Sabouraud dextrose agar (Merck, Darmstadt, Germany) at 4 ^{o}C. All encapsulated strains were thinly encapsulated as determined by Indian ink examination (<0.5 μM). Prior to the experiments, cells were freshly grown in to Sabouraud glucose liquid, washed, counted, heat-killed for 30 minutes at 80°C and resuspended at a concentration of 10^8 per ml.

Capsular components

GXM NIH 37 (serotype A) was isolated and purified as previously described (10). GXM B3502 (serotype D) was a gift from Dr. J.E. Bennett (National Institute of Health, U.S.A.). GalXM and MP fractions were a kind gift from Dr. R. Cherniak (Georgia State University, Atlanta, Georgia, U.S.A.).

Isolation of PBMC

PBMC were isolated by centrifugation on a Ficoll-Paque density gradient (Pharmacia, Sweden). The mononuclear cell layer was then washed and resuspended in RPMI 1640 (Gibco, Gaithersburg, Md) containing 0.1% human serum albumin, to a final concentration of 10^7 cells ml^{-1}.

Stimulation of PBMC by *C. neoformans* and its capsular components

Isolated cells were stimulated with whole cryptococci (10^8 ml^{-1}) or the capsule components (25 μg ml^{-1}) in the presence of 10 μg ml^{-1} polymyxin B sulphate as described by Chaka *et al.* (11). Negative control PBMC were incubated with RPMI 1640 with normal human serum (NHS) in presence of 10 μg ml^{-1} polymyxin B sulphate. Prior to incubation with PBMC (10^6) cryptococci and capsular components were incubated with NHS at 37°C for 30 minutes. After incubation (3, 6 and 18 h), samples were centrifuged, supernatants collected and stored at -70° C prior to cytokine assays.

Cytokine determination

TNF-α, IL-6, IL-8, IFN-γ and IL-10 levels in supernatants were assayed by enzyme linked immunosorbent assay (ELISA) kits (CLB, Amsterdam, The Netherlands), according to the manufacturer's instructions. The detection limits (lowest positive standard) were: TNF-α, 1.4 pg ml^{-1}; IL-6, 0.6 pg ml^{-1}, IL-8, 1 pg ml^{-1}, IFN-γ, 2.5 pg ml^{-1} and IL-10, 1.2 pg ml^{-1}. The IL-1 β ELISA used in the study was developed within our laboratory. The detection limit of the ELISA was 25 pg ml^{-1}. The amount of the various cytokines

produced by the PBMC incubated with polymyxin B and NHS was subtracted from the values obtained with the PBMC stimulated with either the whole cryptococci or the cryptococcal components, both in the presence of polymyxin B.

RESULTS

Cytokine profiles induced by *C. neoformans* strains in PBMC

C. neoformans strains were opsonized with NHS for 30 min. and subsequently incubated with PBMC. TNF-α, IFN-γ, IL-1β, IL-6, IL-8 and IL-10 levels were determined after 3, 6 and 18 h of incubation by ELISA. At t=0, no cytokines could be detected in the fresh culture medium. Control PBMC incubated with RPMI 1640 with NHS in presence of 10 μg ml^{-1} polymyxin B produced background levels of the different cytokines (data not shown). All three strains induced similar patterns of cytokines (Fig.1 A, B, C). After 3 h, high levels of TNF-α were observed, which were strongly diminished after 18 h for strains NIH 37 ($p<0.05$) and W11 ($p< 0.0025$); and to a lesser extent for W22 (not significantly). Low levels of IL-10 were detected after 3 h, which slowly increased during the course of the experiment (significantly for NIH 37: $p<0.05$). IFN-γ was elicited by all the three strains. The cytokine patterns for IL-1β, IL-6 and IL-8 were similar for all strains: constantly high levels of IL-8 and somewhat lower levels of IL-1β and IL-6.

Cytokine patterns evoked by GXM and GalXM in PBMC

GXM NIH 37 (serotype A) and GXM B3502 (serotype D) were utilized in this study. Both types GXM evoked a similar type of cytokine patterns as compared to the whole cryptococci (Fig. 1 and Fig. 2A and 2B). Significant decreases of TNF-α levels ($p< 0.05$ for GXM NIH 37 and B3502), small (not significant) decreases in IFN-γ and increases of IL-10 levels (significant for GXM NIH 37; $p<0.01$) were observed. IL-8 levels induced by GXM from two different serotypes were similar when compared to whole cryptococci, the IL-1β and IL-6 levels were, in general, higher (Fig. 1, Fig 2A and 2B). GalXM, one of the minor carbohydrate components of the cryptococcal capsule, has a completely different structure when compared to GXM. Nevertheless, stimulation of PBMC by GalXM in the presence of NHS, evoked similar cytokine profiles as compared to whole strains and GXM (Fig. 2C).

Cytokine patterns evoked by MP in PBMC

PBMC were finally stimulated with MP1 (Fig. 3A) MP2 (Fig. 3B) and MP4 (Fig. 3C) and cytokine levels were determined after 3, 6 and 18 h. Again similar cytokine patterns as for all the other stimuli studied (whole cryptococci, GXM and GalXM) were elicited by the three mannoprotein fractions: decreases of TNF-α levels ($p < 0.01$ for all three MP) and IFN-γ levels after 18 h of stimulation, and an increase of IL-10 during the course of the experiment. In general, after stimulation with all MP high IL-1β, IL-6 and IL-8 levels were observed, which were constant and similar to the levels induced by GXM.

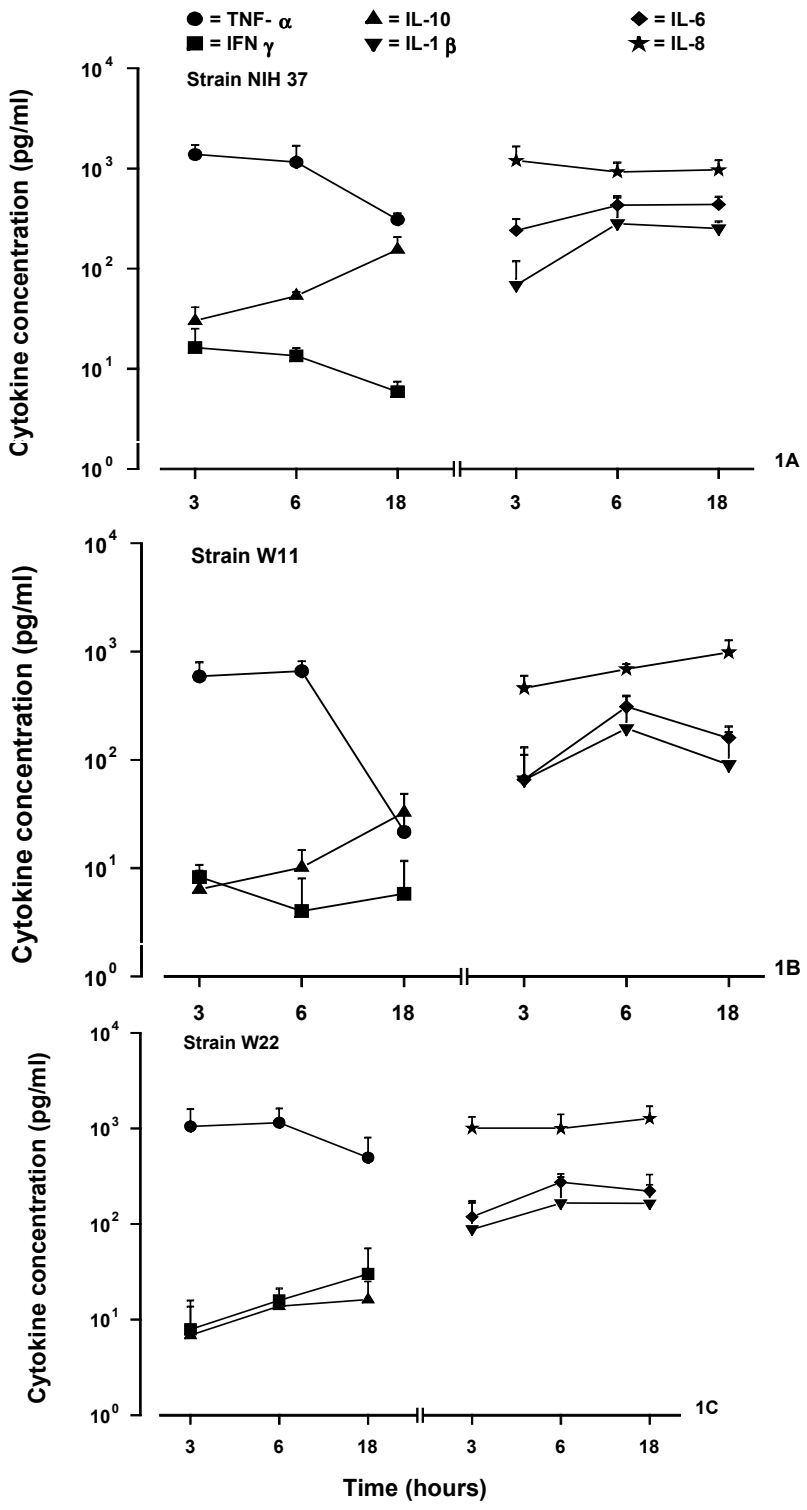

Fig. 1A,B,C. Cytokine profiles induced in PBMC by *C. neoformans.* PBMC (10^6) were incubated with either strain NIH 37 (A), W11 (B) or W22 (C) (all 10^8 cryptococci) for 3, 6 or 18 h in the presence of 10% NPS. Supernatants were collected and the cytokines IFN-γ, TNF-α, IL-1β, IL-6, IL-8 and IL-10 were determined by ELISA. The amount of the various cytokines produced by the PBMC incubated with polymyxin B and NHS alone was subtracted of the values obtained with the PBMC stimulated with whole cryptococci in the presence of polymyxin B. Results are the mean of 3 experiments ± SEM.

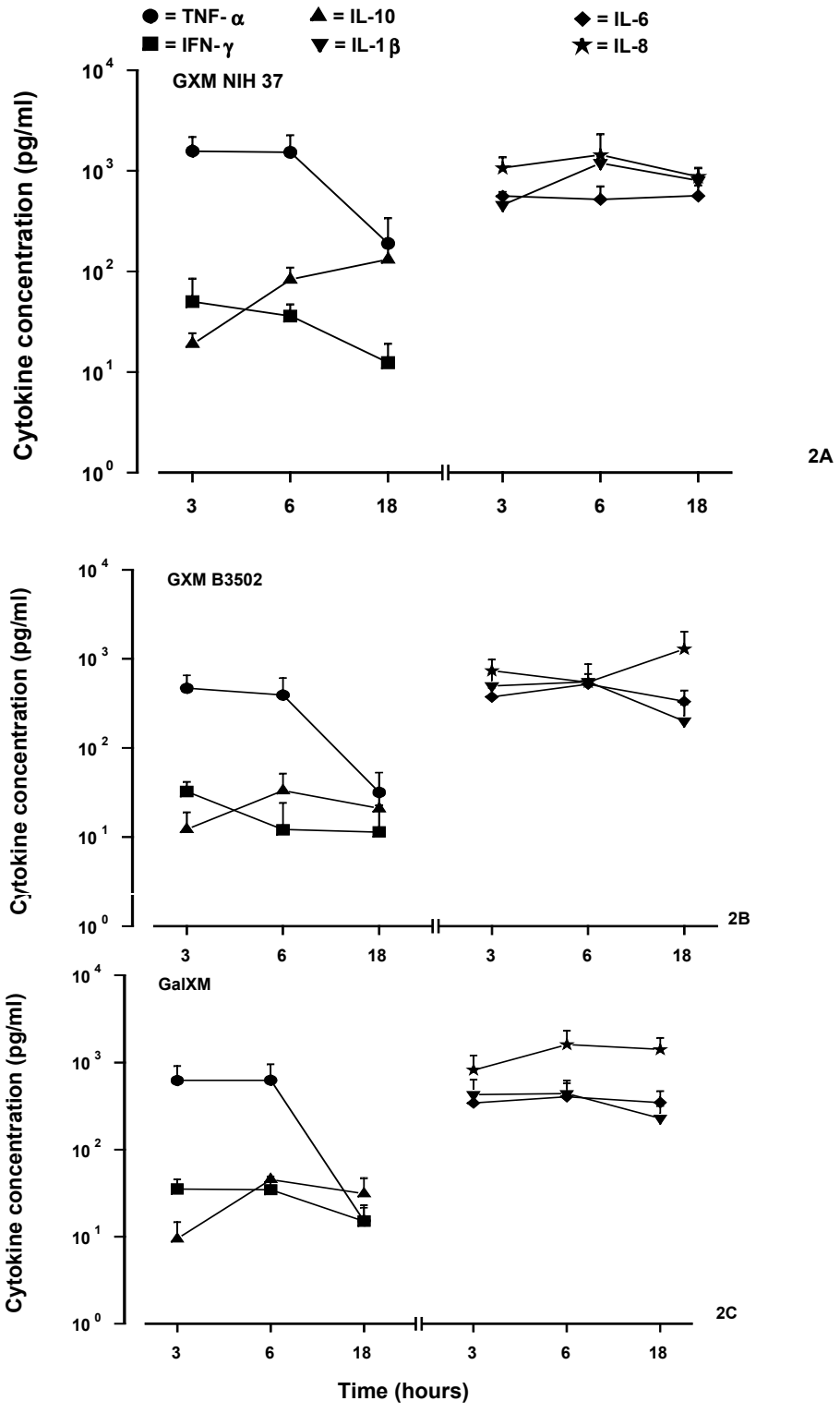

Fig. 2A,B,C. Cytokine profiles induced by GXM or GalXM in PBMC. PBMC (10^6) were incubated with either 25µg ml^{-1} GXM NIH 37 (A), 25µg ml^{-1} GXM B3502 (B) or 25 µg ml^{-1} GalXM (C) for 3, 6 or 18 h in the presence of 10% NPS. Supernatants were collected and the cytokines IFN-γ, TNF-α, IL-1β, IL-6, IL-8 and IL-10 were determined by ELISA. The amount of the various cytokines produced by the PBMC incubated with polymyxin B and NPS alone was subtracted of the values obtained with the PBMC stimulated with either the GXM or GalXM in the presence of polymyxin B. Results are the mean of 3 experiments ± SEM.

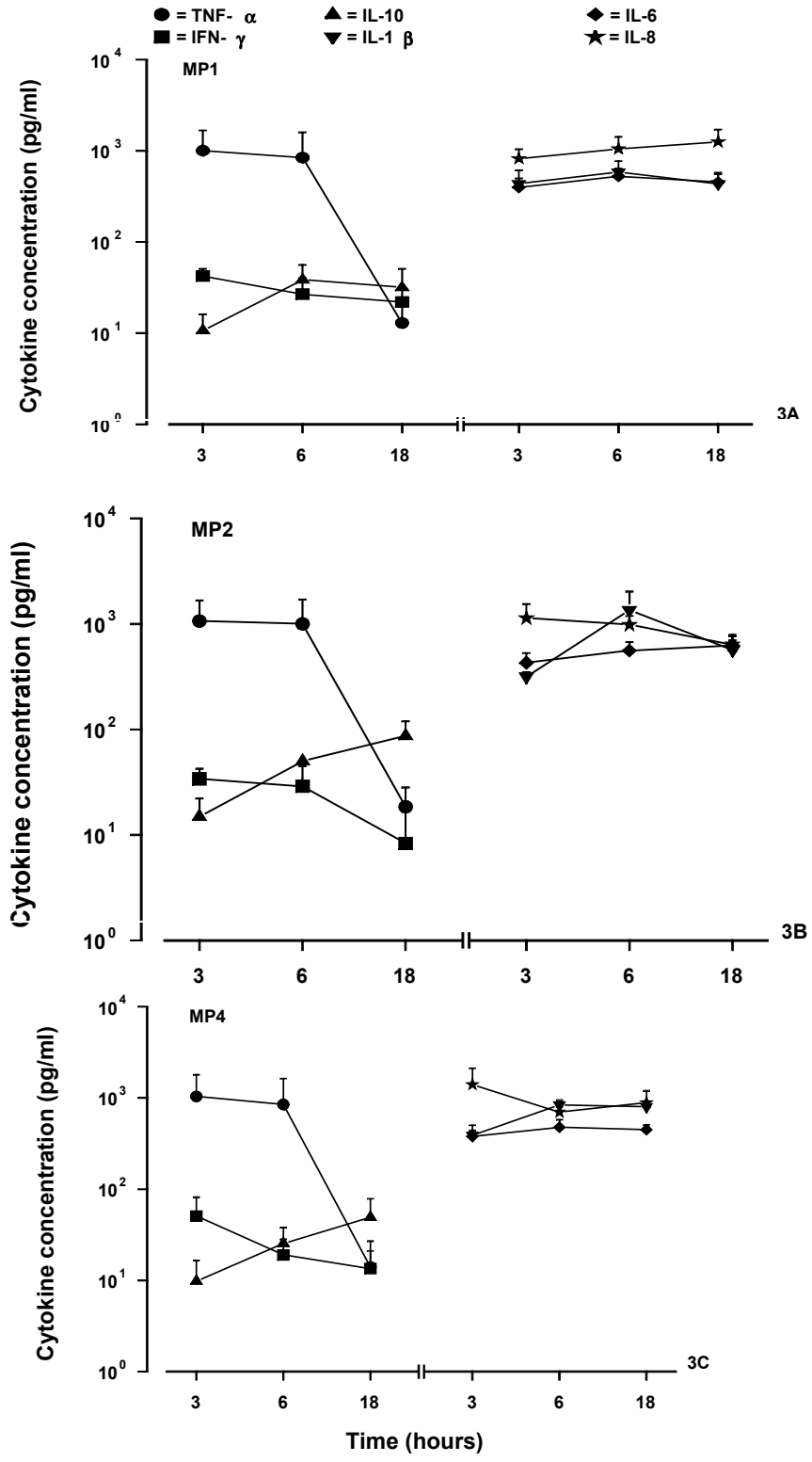

Fig. 3A,B,C. Cytokine profile induced by mannoprotein fractions in PBMC. PBMC (10^6) were incubated with either MP1 (A), MP2 (B) or MP4 (C) (all 25 μg ml^{-1}) for 3h, 6h or 18h in the presence of 10% HPS. Supernatants were collected and the cytokines IFN-γ, TNF-α, IL-1β, IL-6, IL-8 and IL-10 were determined by ELISA. The amount of the various cytokines produced by the PBMC incubated with polymyxin B and NPS alone was subtracted of the values obtained with the PBMC stimulated with MP in the presence of polymyxin B. Results are the mean of 3 experiments ± SEM.

DISCUSSION

Given the ubiquitous presence of *C. neoformans* in the environment, it is very likely that host resistance in normal healthy subjects to infection is very high. Host resistance to cryptococci undoubtedly involves complex interactions between host defense mechanisms and yeasts. The immunoregularory effects of *C. neoformans* would be mediated in part through selective induction of phagocyte derived cytokines. Here, we analyzed the cytokine patterns evoked by *C. neoformans* and its isolated capsular components in PBMC.

We showed that *C. neoformans* and its different capsule components induce TNF-α release by PBMC (11). The results of the present study show that different strains of *C. neoformans* and its purified capsular components stimulate expression of pro-inflammatory cytokines IFN-γ, IL-1β, IL-6 and IL-8 and anti-inflammatory cytokine IL-10. Whole cryptococci and all cryptococcal components studied induced a very similar pattern of cytokine release. The time kinetics of release for the various cytokines, however, were different with IFN-γ and TNF-α showing early release at 3 hours with levels dropping at 18 hours. After 3 hours of stimulation the release of IL-10 was low, but levels steadily increased over time.

The observed similarity of the cytokine patterns induced by the various strains and components of *C. neoformans* was unexpected. GXM, GalXM and MP differ in protein content, amino acid composition, and monosaccharide composition (10;12). Moreover, cytokine induction in PBMC by GXM depends on the presence of an intact complement system, whereas for GalXM and MP this process is partially facilitated by human mannose binding lectin, a heat stable component (8;11). Part of the observed and not foreseen similarity in cytokine release might be caused by indirect effects. For example, the observed cytokine profile, might be strongly influenced by the autocrine-paracrine cytokine network, since the presence of e.g. high levels of TNF-α induces IL-10 independent of the type of stimulus (13). Moreover, polysaccharides, glycolipids, and glycoproteins with a large variety of carbohydrate structures are known to induce cytokines in PBMC (14-16). GXM and GalXM are completely composed of carbohydrate and the model proposed for cryptococcal MP, a protein backbone substituted with a large number of short oligosaccharides, suggests that these molecules are seen by the host-defense system more as carbohydrate than as a protein.

The different cytokines induced by whole cryptococci and by the components can either play a beneficial or a detrimental role in host defense against *C. neoformans*. *In vitro* studies have shown that the antiphagocytic potential of *C. neoformans* could be overcome by macrophages activated by TNF-α and granulocyte macrophage colony stimulating factor (GM-CSF) (17), and that administration of neutralizing antibody against TNF-α was deleterious for *C. neoformans* infected mice (18). In another study, the ability to clear a pulmonary *C. neoformans* infection in mice was shown to correlate with high IFN-γ

secretion by lung associated lymph nodes (19), whereas decreased levels of IL-6 were associated with an abrogation of pulmonary clearance of *C. neoformans* in mice (20). IL-10 probably has a detrimental role in host-defense against cryptococcal infection. This cytokine has been shown to inhibit synthesis of TNF-α, IL-1β and IL-6 in mononuclear cells, it down regulates Class II MHC expression on macrophages and may alter T cell responses through inhibition of TNF-α (21). In this study all strains and the capsular components of *C. neoformans* induced IL-10 release by PBMC. Vecchiarelli *et al*., demonstrated that GXM prepared from a *C. neoformans* type A strain induced IL-10 by human monocytes (22). They also showed that IL-10 was involved in regulating TNF-α and IL-1β secretion by monocytes in response to *C. neoformans*, and Levitz *et al*. demonstrated the ability of IL-10 to inhibit PBMC responses to *C. neoformans* (23).

In conclusion, the time kinetics of cytokine release in this study indicate an autocrine-paracrine network regulation of the cytokines, with proinflammatory cytokines TNF-α and IFN-γ being released early while IL-1β, IL-6 and IL-8 showed a more sustained release and IL-10 clearly increasing over time. We did not observe the tendency for a particular cryptococcal strain or capsular component to induce a type 1 or a type 2 cytokine response, but in the first 3 hours a more type 1 pattern was observed. The time kinetics of the cytokine induction by *C. neoformans* and its capsular components might therefore provide a time-window that facilitates the induction of cellular immunity, which is the corner stone of host-defense against cryptococci.

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CHAPTER 6

Production of CC chemokines by HIV-infected human macrophages stimulated with *C. neoformans* and its capsular component glucuronoxylomannan

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SUMMARY

The object of this study was to analyze the ability of *C. neoformans* and its major capsule component glucuronoxylomannan (GXM) to induce the C-C chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and regulated upon activation normal T-cell expressed and secreted (RANTES) in primary HIV-infected human macrophages. Reverse transcriptase-polymerase chain reaction was used to detect mRNA of these chemokines after stimulation of both HIV-infected and uninfected macrophages with *C. neoformans* or GXM. In addition, protein levels of these chemokines were determined by ELISA. In contrast to RANTES, MIP-1α and MIP-1β levels were higher in the HIV-infected *C. neoformans*- or GXM-stimulated macrophages as compared to stimulated uninfected cells. These data show that both HIV and *C. neoformans* elicit the production of C-C chemokines by macrophages allowing mononuclear cell recruitment to tissue.

INTRODUCTION

C. neoformans is an encapsulated yeast like fungus and the leading cause of mycological infections of the central nervous system (CNS) in patients with impaired cell-mediated immunity, especially in those with AIDS. Despite antifungal therapy, between 10-25% of cryptococcosis patients die during initial therapy, and between 30-60% succumb within 12 months (1). In order to design improved intervention strategies, better understanding of the host defense mechanisms against these yeasts is needed.

The capsule of *C. neoformans* is composed of at least three well characterized antigens: a capsular polysaccharide glucuronoxylomannan (GXM), a minor polysaccharide galactoxylomannan (GalXM), and mannoproteins (MP) (2). GXM, the major capsule polysaccharide of *C. neoformans,* is known to be anti-inflammatory, anti-phagocytic, and immunosuppressive (1). Moreover, GXM can induce IL-8 production in cultured human microglia cells (3), and has been described to interfere with neutrophil migration towards the CNS in cryptococal meningitis patients, despite the presence of chemokines like IL-8 in the CSF (4;5).

Lee *et al*. analyzed, postmortem, brain tissue from 21 patients with cryptococcal meningoencephalitis (CME). Distribution of GXM in regions that are known to localize HIV gp41 antigen raised the possibility of a symbiotic relationship between HIV and *C. neoformans* (6). In HIV encephalitis massive infiltration of macrophages into the brain compartment can be detected and HIV-1-infected macrophages and microglia have been identified as the virus reservoirs in brain tissue (7). In AIDS-related cryptococcal meningoencephalitis brain macrophages and microglia were identified as principal inflammatory cells (8). The means by which these cells are attracted into the brain compartment have not been elucidated, but chemokines have been suggested to be involved (9). Chemokines constitute a super gene family of small inducible peptides that

possess potent chemotactic activity for leukocytes. Two major chemokine families can be distinguished based on the position of conserved cysteine residues in the molecules. The C-C chemokines include among others, MIP-1α and MIP-1β and RANTES. The C-C chemokines, in general, do not act on neutrophils but attract monocytes, eosinophils, basophils, and lymphocytes by binding to specific CC chemokine receptors (CCR) on these cells, including CCR5 (10). CCR5 also functions as a coreceptor for macrophage-tropic, nonsyncytium-inducing strains of HIV. MIP-1α and MIP-1β and RANTES suppress replication of macrophage-tropic HIV strains in vitro, presumably by competitive inhibition of HIV attachment to CCR5 (11). In a mouse model, the chemokines MIP-1α and MCP-1 have been described to play a major role in leukocyte recruitment into the lungs following infection with *C. neoformans* (12-14). In the present study MIP-1α and MIP-1β and RANTES gene expression and release in both HIV-infected and uninfected macrophages stimulated with *C. neoformans* and GXM was investigated.

MATERIAL AND METHODS

Cryptococcus neoformans

Encapsulated *C. neoformans* (NIH 37) was used throughout this study. The organisms were obtained from the National Institute of Health (NIH, Bethesda, MD). NIH 37 is a thinly encapsulated clinical isolate (serotype A) from cerebrospinal fluid obtained by K.J. Kwon-Chung. The yeast cells were maintained on Sabouraud dextrose agar (Merck, Darmstadt, Germany) at 4°C. After harvesting, the cryptococci were heat-killed for 30 min at 80°C.

Cryptococcal capsular polysaccharide

Purified GXM, obtained as previously described (15) from *C. neoformans* serotype A (ATCC 62066), was a generous gift from Prof. Dr. R. Cherniak, Georgia State University (Atlanta, GA). Experiments were carried out aseptically to avoid endotoxin contamination. Preliminary experiments were performed in the presence and absence of Polymyxin B sulphate (Sigma) at a final concentration of 10 μg/ml. This concentration of polymyxin B sulphate completely abolishes stimulatory effects of endotoxin from *E.coli* O111:B4 at concentrations up to 100 ng/ml.

HIV-1 infection of macrophages

Peripheral blood mononuclear cells were isolated from heparinized blood from HIV-1-, HIV-2-, and hepatitis B-seronegative donors and obtained on Ficoll-Hypaque density gradients. Cells were washed twice and monocytes were purified by countercurrent centrifugal elutriation. Cells were >98% monocytes by criteria of cell morphology on May-Grünwald-Giemsa-stained cytosmears and by nonspecific esterase staining using α-naphtylacetate (Sigma Chemical Co., St. Louis, MO) as substrate. Monocytes were

cultured in suspension at a concentration of 2 x 10^6 cells/ml in Teflon flasks (Nalgene, Rochester, NY) in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated human AB serum negative for anti-HIV antibodies, 10 µg/ml gentamicin, and 10 µg/ml ciprofloxacin (Sigma). After 7 days monocyte-derived macrophages (MDM) were recovered from the Teflon flasks and infected with HIV-1_{Ba-L} at a multiplicity of infection of 0.01 for two hours. HIV-infected and mock-infected MDM were washed twice to remove unbound virus and cultured in Teflon flasks for an additional five days to establish a chronic infection. Then, MDM were washed and added in a 24-well plate.

Stimulation of HIV-infected macrophages

HIV infected and uninfected macrophages (both 3 x 10^5/well) were stimulated with buffer (negative control), or *C. neoformans* (3 x 10^6/well) or GXM A (25 µg/ml), in the presence of 10% HPS. After 8 h incubation samples were collected and stored at -70^0C prior to chemokine-ELISA's and stimulated macrophages were lysed for the purpose of RT-PCR detection of chemokines.

Chemokine determination by RT-PCR and ELISA

Macrophages were homogenized and lysed in 1 ml TRIzol (Life Technologies Gaithersburg, MD) according to the manufacturer's guidelines. When lysates of all time points were obtained, total RNA was isolated. Total RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and 1 µg of RNA was used for the synthesis of complementary DNA and PCR reactions were performed as described previously (16). For semi-quantification every primer pair was tested at different cycle numbers to determine the linear range. GAPDH mRNA levels were measured at 20 cycles, whereas cDNA had to be subjected to 23 cycles to be in the linear range to detect MIP-1α, MIP-1ß, and 26 cycles for RANTES. Aliquots of 5 µl of the biotinylated PCR product were semi-quantitatively analyzed using a fluorescent digoxigenin detection ELISA kit (Boehringer Mannheim) according to manufacturer's protocol as described previously (16). All data were normalized against GAPDH mRNA levels. The 5'-3' sequences of the oligonucleotide primers and probes in reverse transcriptase polymerase are: GAPDH (195bp), sense CCATGGAGAAGGCTGGGG, antisense CAAAGTTGTATGGATACC, probe CTGCACCACCAACTGCTTAGC; MIP-1α (333 bp) sense TGCATCACT-GCTGCTGACACG antisense CAACCAGTCCATAGAAGAGG probe CTGACTA-TTTGAGACGAGC; MIP-1ß sense CCAAACCAAAAGAAGCAAGC antisense AGAAACAGTGACAGTGGACC probe ACATCTCCTCCATACTCAGG; RANTES sense CTTTGTCACCCGAAAGAACC antisense GTTTCATCATGTTGGCCAGG probe TTGCTCTTGTCCTAGCTTGG. MIP-1α, MIP-1β, and RANTES levels in supernatants were assayed by enzyme linked immunosorbent assay (ELISA) kits (MIP-1α, MIP-1β: R&D Systems Minneapolis, Mn. RANTES Endogen, Woburn, MA), according to the manufacturer's instructions. The absorbance at 450 nm was measured with a micro plate reader (Biorad, Tokyo, Japan).

RESULTS

C-C chemokines mRNA production by HIV-infected macrophages stimulated with *C. neoformans* and GXM

A 3-fold induction of mRNA levels of MIP-1α was seen after stimulation of uninfected macrophages with *C. neoformans* or GXM. HIV-1 infection of macrophages increased mRNA levels of MIP-1α 2-fold. *C. neoformans* or GXM stimulation of HIV-infected macrophages upregulated mRNA levels of MIP-1α 3.3-fold, as compared to unstimulated HIV-infected macrophages (Fig. 1A).

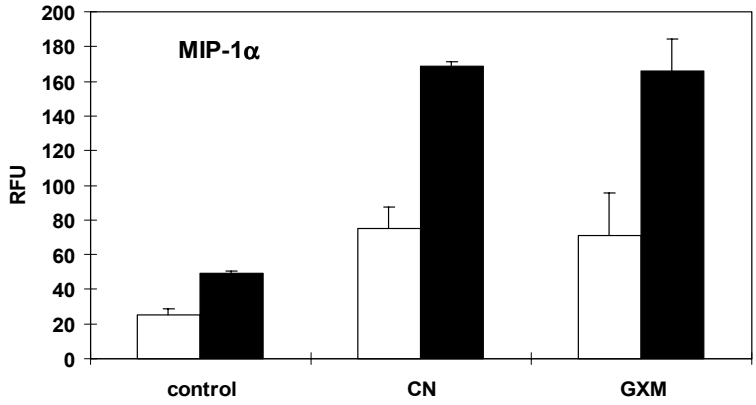

Figure 1A. mRNA expression levels, expressed in relative fluorescence units (RFU) of MIP-1α in uninfected (open bars) and HIV-infected (solid bars) macrophages. The means plus standard errors of the mean (SEM) of three experiments are shown.

HIV-1 infection of the macrophages increased mRNA levels of MIP-1β 1.4-fold. Stimulation of uninfected macrophages with *C. neoformans* or GXM showed a 4-fold induction of mRNA levels of MIP-1β. However, a 7.2-fold induction was seen after stimulation of HIV-infected macrophages with *C. neoformans* or GXM (Fig 1B).

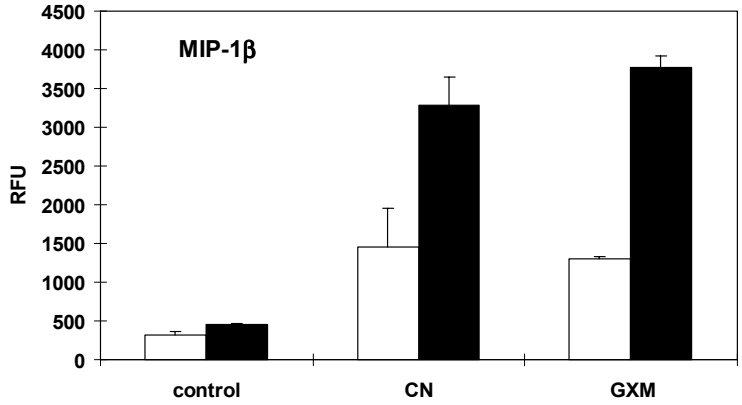

Figure 1B. mRNA expression levels, expressed in relative fluorescence units (RFU) of MIP-1β in uninfected (open bars) and HIV-infected (solid bars) macrophages. The means plus standard errors of the mean (SEM) of three experiments are shown.

In contrast to mRNA levels of MIP-1α and MIP-1β, no effect of stimulation with *C. neoformans* or GXM of uninfected macrophages on mRNA levels of RANTES could be detected. HIV-1 infection of macrophages increased mRNA levels of RANTES 1.4-fold. Stimulation of HIV-infected macrophages with GXM resulted in a 1.8-fold induction of mRNA RANTES levels as compared to unstimulated HIV-infected cells (Fig 1C).

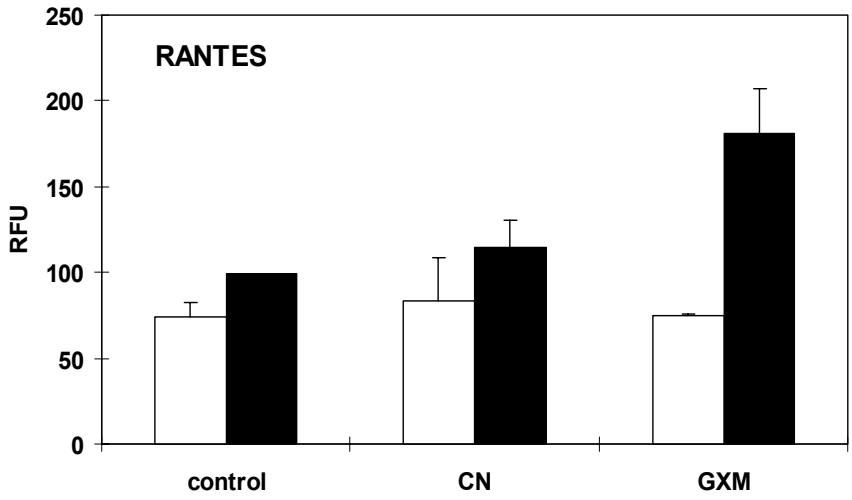

Figure 1C. mRNA expression levels, expressed in relative fluorescence units (RFU) of RANTES in uninfected (open bars) and HIV-infected (solid bars) macrophages. The means plus standard errors of the mean (SEM) of three experiments are shown.

Induction of protein levels of C-C chemokines by HIV-infected macrophages stimulated with *C. neoformans* and GXM

Next, we investigated whether mRNA levels of the three C-C chemokines reflected protein levels of these molecules. Therefore, ELISA's for MIP-1α, MIP-1β, and RANTES were performed (Table 1). Unstimulated uninfected macrophages produced 361 pg/ml MIP-1α. HIV infection of macrophages increased this level 3.2 fold. GXM-stimulated HIV infected macrophages produced 7160 pg/ml, a 6-fold induction as compared to unstimulated HIV-infected cells. MIP-1β levels in unstimulated uninfected macrophages were comparable with the levels of HIV-infected unstimulated macrophages (319 pg/ml, 329 pg/ml respectively). GXM enhanced the production of MIP-1β 7.4 fold, whereas cryptococci increased this level to 679 pg/ml (a 2-fold induction), both as compared with unstimulated HIV-infected cells. mRNA levels for RANTES were 2849 pg/ml in unstimulated uninfected macrophages. These levels increased after stimulation of uninfected cells with *C. neoformans* or GXM (1.2 and 1.6 fold respectively). Stimulation of HIV-infected cells with GXM only increased RANTES levels as compared to HIV infected unstimulated macrophages 1.2 fold.

Table 1: Protein levels of MIP 1α, MIP 1β, and RANTES of *C. neoformans* and GXM stimulated HIV- and uninfected human macrophages (pg/ml). Data are the mean of a representative experiment (plus standard error), performed in triplicates.

	MIP-1α		**MIP-1β**		**RANTES**	
Stimulus	control	HIV	control	HIV	control	HIV
Control	361 (62)	1158 (13)	319 (62)	329 (46)	2849 (399)	4723 (560)
CN	687 (128)	2752 (380)	545 (91)	679 (142)	3312 (451)	5087 (594)
GXM	318 (36)	7160 (770)	121 (14)	2442 (467)	4465 (513)	5637 (687)

DISCUSSION

Cryptococcal meningoencephalitis (CME) in the setting of AIDS is frequently distinguished by a marked lack of an inflammatory response (17). AIDS patients with cryptococcal meningitis have been reported to have low CSF leukocyte counts, despite high levels of the neutrophil-attracting chemokine interleukine–8 in the cerebrospinal fluid (4). Human polymorphonuclear neutrophils (PMN) have been reported to effectively kill *C. neoformans* (18). However, PMN are not found in brain parenchyma of these patients suggesting that the process of host cell recruitment to the CNS is carefully regulated by *C. neoformans*. Indeed, it has been shown that a low leukocyte count in cryptococcal meningitis patients affects prognosis negatively (19).

In this and in other studies (16), it was shown that HIV-1 infection of macrophages increases mRNA levels of MIP-1α, MIP-1β, and RANTES. Moreover, both HIV infection and *C. neoformans* or GXM stimulation of macrophages resulted in an additional enhancement of chemokine expression. Huang *et al.* showed the same pattern of chemokine release in Peripheral Blood Mononuclear Cells from persons with and without HIV infection (11). Furthermore we show that mRNA levels of MIP-1α, MIP-1β, and RANTES roughly reflect protein levels of the three chemokines. These data suggest that the lack of inflammatory response is not caused by defective chemokine release. Apparently high levels of chemokines fail to attract sufficient numbers of effective leukocytes to *C. neoformans* infected places.

Paucity of inflammatory cells despite sufficient amounts of chemokines could be explained by more specific inhibitory mechanisms as are described for neutrophils. Dong and Murphy described that cryptococcal polysaccharides can cause shedding of L-selektine from the surface of neutrophils in mice (20) and GXM binds to CD18 adhesion

molecules thereby blocking interaction of activated neutrophils with ligands on the endothelium (21).

Animal models show that chemokines play an important role in cryptococcosis. The chemokines MIP-1α and MCP-1 have been described to play a major role in leukocyte recruitment into the lungs following infection with *C. neoformans* (12-14). Recently, it was shown in a mouse model that a Th1 cell mediated inflammatory response that requires chemokines for the recruitment of effector cells is needed for clearance of *C. neoformans* from the CNS (22).

Acapsular *C. neoformans* enter the body via the respiratory tract and in the lung cells of the host defense system phagocytize them (1). In cases of dissemination from the lungs, cryptococci are able to escape local (impaired) innate and adaptive immunity and gain access to the bloodstream. The production of C-C chemokines by stimulated HIV-infected macrophages suggests the possibility that monocytes/macrophages are specifically attracted from the bloodstream into the brain parenchyma. Subsequently this may result in the delivery of yeasts into the brain tissue by *C. neoformans* infected-monocytes/macrophages as intracellular survival of cryptococci in macrophages has been described (23). Very recently, not only intracellular survival but even replication of *C. neoformans* in macrophages has been described (24). In HIV encephalitis massive infiltration of macrophages into the brain compartment can be detected and HIV-1 infected macrophages and microglia have been identified as the virus reservoirs in brain tissue (7). The CC chemokine attracted cells from the mononuclear system could be responsible for the maintenance of both HIV and *C. neoformans* reservoir in the CNS.

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CHAPTER 7

Does the capsular component glucuronoxylomannan of *Cryptococcus neoformans* impair transendothelial migration of leukocytes in patients with cryptococcal meningitis?

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The encapsulated yeast like fungus *Cryptococcus neoformans* is the leading cause of mycological infection in the central nervous system (CNS) in patients with compromised cell-mediated immunity (1). Before, we demonstrated that the cerebrospinal fluid (CSF) of patients with cryptococcal meningitis contains high levels of the neutrophil chemoattractant IL-8 despite the fact that the CSF contains few neutrophils (2). The cryptococcal capsular polysaccharide glucuronoxylomannan (GXM) is both present in serum and in CSF of patients with cryptococcal meningitis, and GXM is known to interfere with neutrophil migration (3). We demonstrated *in vitro* that GXM is capable of inducing the production of IL-8 by brain cells, whilst GXM also prevents neutrophils from migrating towards IL-8 (4). Consequently, a high ratio of GXM in serum/CSF should correlate with a low CSF leukocyte cell count in patients with cryptococcal meningitis. Therefore, we compared retrospectively the GXM titers in serum and CSF with the CSF leukocyte cell counts of 35 Dutch HIV-infected patients with a culture-proven diagnosis of cryptococcal meningitis between 1986 and 1996. Antigen titers had been measured with commercial kits routinely used for diagnostic detection of cryptococcal antigen, (mainly Murex Cryptococcus Test, Kent, UK), and were obtained within 5 days of CSF leukocyte cell counts. Since GXM can attract neutrophils itself (4), the GXM concentration gradient over the blood brain barrier, expressed as the ratio of titers in serum versus CSF, is expected to be more critical to the CSF cell count than absolute GXM concentrations. Figure 1 demonstrates a significant inverse correlation between the (log) GXM ratio and the (log) CSF leukocyte cell count in patients with cryptococcosis. (Correlation coefficient of log values: -0.54, n=35; two-sided $P<0.001$). These data suggest that the *in vitro* finding of interference of GXM with neutrophil migration may indeed represent a pathogenitic mechanism in cryptococcal meningitis.

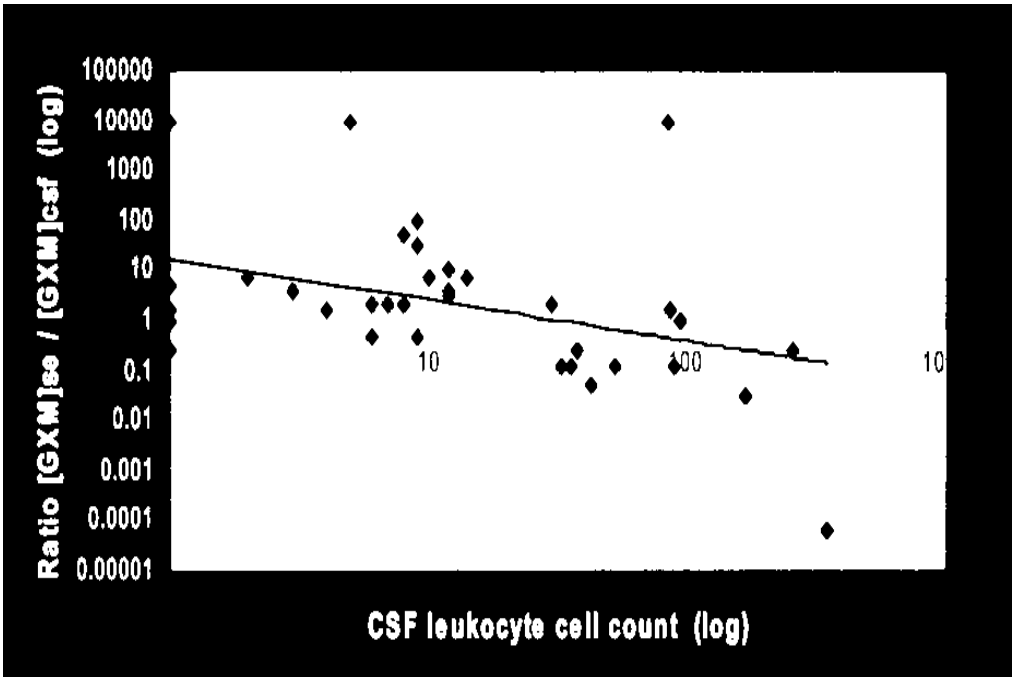

Figure 1

Inverse correlation between the ratio of cryptococcal glucuronoxylomannan titers in serum ([GXM]se) over cerebrospinal fluid ([GXM]csf) and the CSF leukocyte cell count in 35 patients with cryptococcal meningitis.

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CHAPTER 8

Potent Inhibition of Neutrophil Migration by Cryptococcal Mannoprotein-4 -induced Desensitization

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ABSTRACT

Cryptococcal capsular antigens induce the production of pro-inflammatory cytokines in patients with cryptococcal meningitis. Despite this, their cerebrospinal fluid typically contains few neutrophils. Capsular glucuronoxylomannan (GXM) is generally considered to mediate the inhibition of neutrophil extravasation. In the current study culture supernatant harvested from the non GXM-producing strain CAP67 was found to be as potent as supernatant from wild-type strains in preventing migration. We identified capsular mannoprotein-4 (MP-4) as the causative agent. Purified MP-4 inhibited migration of neutrophils toward platelet-activating factor (PAF), IL-8 and fMLP, probably via a mechanism involving chemoattractant receptor cross-desensitization as suggested by its direct chemotactic activity. Supporting this hypothesis, MP-4 elicited Ca^{2+} transients that were inhibited by pre-incubation with fMLP, IL-8 or C5a, but not PAF - and vice versa. Moreover, MP-4 strongly decreased the neutrophil surface expression of L-selectin and induced shedding of TNF receptors p55/p75, while CD11b/18 increased. Finally, MP-4 was clearly detectable in both serum and cerebrospinal fluid of patients suffering from cryptococcal meningitis. These findings identify MP-4 as a novel capsular antigen prematurely activating neutrophils and desensitizing them toward a chemoattractant challenge.

INTRODUCTION

Neutrophil infiltration into the central nervous system is a dual-edged sword in that it protects the tissue from infection and injury but is also detrimental to the host. In diseases such as bacterial meningitis (BM) or cerebrovascular ischemia there is evidence that a high influx of polymorphonuclear neutrophils (PMN) plays an adverse role in the pathogenesis of neurological damage (1;2). In BM, the phagocytic capacity of PMN in the cerebrospinal fluid (CSpF) is often insufficient and the harmful effects of their cytotoxic products may outweigh their beneficial effects (3). Accordingly, it is now assumed that adjunctive therapeutic strategies with anti-inflammatory agents may favor neurological recovery (4-6). Interestingly, the opportunistic fungus *Cryptococcus neoformans* has developed a means of infection evoking only minimal PMN infiltration.

Disseminated cryptococcosis is characterized by the presence of high levels of capsular glucuronoxylomannan (GXM), galactoxylomannan (GalXM), mannoprotein-1 (MP-1), and MP-2, in the CSpF and serum of affected patients (7;8). Titers in both serum and CSpF from AIDS patients reach levels equivalent to several hundred micrograms per milliliter, in exceptional cases rising to 20 mg/mL (9). GXM, GalXM and MP have been shown to induce the production of the early pro-inflammatory cytokines TNF-α and IL-1β by peripheral blood monocytes (10-12) and PMN (13) in the presence of pooled human serum (PHS). TNF-α then plays a pivotal role (14) in

initiating a protective cell-mediated immune response. In healthy subjects, TNF-α enables the host to overcome the anti-phagocytic properties of the fungus, which usually suffices to clear systemic infection (15). In patients in whom this accurate response is hampered, the disease disseminates and induces inflammation. This reaction theoretically could be a very purulent one since the induced TNF-α has several properties that strongly promote PMN extravasation. First, TNF-α can cause profound systemic vasodilatation and subsequent hypotension via the induction of prostaglandins (16). Hypotension is a prerequisite to allow the transient L-selectin (CD62L)-mediated adherence of leukocytes to endothelial cells (17-19). In addition, TNF-α and IL-1β can induce the expression of adhesion molecules on endothelial and glial cells (4). Furthermore, TNF-α and IL-1β activate mononuclear cells, endothelial cells, and astrocytes to produce IL-8 (20) in the brain of patients with meningitis.

Despite the elevated IL-8 CSpF/serum ratio (13;21) and the elevated serum levels of TNF-α and IL-1β, the CSpF of patients with cryptococcal meningitis typically contains few mononuclear cells and virtually no PMN. To date GXM is considered to account for this inhibition of leukocytosis. In a mouse model study, Dong and Murphy showed that the inhibition of PMN infiltration elicited by intravenous administration of the cryptococcal culture filtrate (CneF) is due mainly to GXM (22). Using a modified Boyden chamber, we previously demonstrated that GXM significantly inhibited PMN migration toward IL-8 (23). In addition, we confirmed Dong and Murphy's earlier observation that GXM mediates the chemotactic activity for PMN of whole encapsulated yeast cells and unfractionated CneF derived from these cells (24). Moreover, we found a significant inverse correlation between the GXM ratio (serum/CSpF) and the CSpF leukocyte count in patients with cryptococcosis (25). More recently, we showed that GXM delays translocation of PMN across the blood-brain barrier in a rabbit BM model (26). Therefore, the initial aim of this study was to investigate the molecular mechanism by which GXM prevents PMN migration toward chemoattractants.

Several studies have investigated the mechanisms regulating the passage of leukocytes through endothelial cells and their infiltration at inflammatory foci. The exact nature of the signaling mechanisms in brain inflammation still remains to be elucidated but undoubtedly involves TNF-α and IL-1β, chemoattractants (IL-8, PAF), as well as the expression of adhesion molecules and proteases that together promote cell recruitment and vascular permeability (6). Initially, localized inflammation results in hypotension allowing selectin-mediated tethering and rolling of PMN along the vessel wall (17;18). Rolling precedes a further functional up-regulation of PMN following exposure to pro-inflammatory cytokines and chemoattractants (27), resulting in firmer integrin-mediated adherence and shedding of CD62L (17;27). Further stimulation of PMN phenotypically high in CD11b/CD18 (CR3, Mac-1)

initiates migration through the interendothelial junctions (28). Finally, chemotactic gradients guide PMN to the site of infection.

Currently, the discrepancy between raised cytokine levels and hampered PMN influx into the brains of patients is explained by the observation that GXM is able to induce shedding of CD62L and TNF-α receptor (TNF-R) molecules from PMN (29). The key observation underlying this paper, i.e. a quantitatively similar prevention of PMN migration by CneF harvested from GXM-producing and non-producing (ΔCneF) strains, seriously questions the opinion that GXM is the sole cryptococcal antigen preventing extravasation. Therefore, we set out to characterize the component of ΔCneF responsible for the inhibition. To assess this, we first optimized the current protocol for isolating capsular antigens and purified the most important constituents of ΔCneF (GalXM, MP-1, MP-2 and MP-4) to near homogeneity (30). We found that MP-4 was primarily responsible for the inhibition of PMN migration. We then investigated the intrinsic chemotactic capacity of MP-4 and analyzed its ability to influence the expression of PMN surface-receptors involved in PMN migration. As a result of the action of MP-4 the surface expression of CD62L was down-regulated and both TNF-R p55 and p75 were shed into the surrounding medium. Furthermore, MP-4-induced signaling caused Ca^{2+} transients that could regulate inflammatory reactions by desensitizing chemoattractant receptors.

MATERIALS AND METHODS

Cryptococcal strains and antigen preparations

The cryptococcal culture filtrate antigens ΔCneF and CneF were prepared from cultures of the non-GXM-producing *C. neoformans* mutant strain, CAP67 (E.S. Jacobson, Medical College of Virginia), or the isogenic capsular strain NIH B3501, respectively. Inocula (10^8 colony forming units) of these strains were added to 500 ml RPMI-1640 supplemented with 10 μg/ml gentamycin and allowed to propagate for 5 days at 37°C. Next, supernatant fluids were isolated by centrifugation and concentrated by ultra-filtration (Stirred Cell Concentrator Millipore, 3.5 kDa cut-off). The concentrated (Δ)CneF was filtered sterilized and adjusted to 0.1 mg of protein per ml as determined by the BCA (Pierce; Rockford, Illinois, USA) protein concentration assay. (Δ)CneF used in the assays was diluted threefold. Mannoproteins were purified from ΔCneF as described recently (30). Briefly, ΔCneF was dialyzed against excess mQ water, freeze-dried and applied to a 100 ml Con A Sepharose-4B column. The column was washed and stepwise eluted with α-methyl-D-mannose pyranoside (αmDm). GalXM flows through this column, whereas MP-1 and -2 are eluted at 0.2 M and MP-4 at 0.4 M αmDm. These fractions were concentrated (MP-4) or further separated (MP-1 and -2) by DE52 or source Q anion exchange columns. All purified components were finally dialyzed against excess PBS and kept frozen at -20°C.

Maintenance of endotoxin-free conditions

Preparations of the various cryptococcal components were negative for endotoxin contamination using a Limulus assay (Kabi Diagnostica, Mölndal, Sweden) with a sensitivity of 100 pg/ml *E. coli* LPS. Nevertheless, all experiments were carried out at least once in the presence of 10 μg/ml polymyxin B sulfate in order to neutralize any undetected LPS contamination.

Isolation of human PMN

PMN were isolated as previously described (31). Briefly, blood from healthy volunteers was collected into Vacuette tubes containing sodium heparin, diluted with an equal volume of pyrogen-free PBS and centrifuged through a gradient of Ficoll and Histopaque. PMN were collected from the Histopaque phase, briefly shocked with mQ water, washed and suspended at 5 x 10^6 cells/ml in RPMI supplemented with 0.05% human serum albumin (RPMI-HSA).

Transwell assay for measurement of chemotaxis

PMN were labeled by incubating them for 30 min. at 20^{o}C with 3.3 μM 2',7'-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxymethyl ester (BC-ECF AM; Molecular Probes Europe, Leiden, Netherlands) in RPMI-HSA. Cells were washed and suspended in HBSS-HSA; 200 μl aliquots were incubated with an equal volume of each of the cryptococcal antigens being assessed for chemoattractive activity or buffer control for 30 min. at 37^{o}C under constant agitation. Just prior to the end of this incubation transwell filters (pore size 3.0 μm; Costar type 3415, Cambridge, MA) were pre-wetted in HBSS/HSA. To each bottom compartment we added 600 μl of the indicated chemoattractants: human recombinant IL-8 (10 nM; Pepro Tech Inc., Rocky Hill, NJ-USA); fMLP (10 nM; Sigma Chem. Co., St. Louis, MO-USA) or PAF (platelet activating factor, 100 nM; Calbiochem, Cambridge, MA-USA). Filter compartments containing 100 μl of PMN suspension were placed on top of the bottom compartments. Migration was allowed to proceed for 1h at 37^{o}C (5% CO_2) and analyzed by reading the fluorescence of the wells with a Cytofluor II multi-well Plate-reader (PerSeptive Biosystems; Farmingham, MA-USA) equipped with a 485 nm excitation and a 530 nm emission filter. Control wells containing BCECF-labeled cells were included to obtain the maximal fluorescence value. The fluorescence values of the samples are expressed relative to this maximum. Fluorescence was linear with cell number with a detection limit of 2,500 cells (1% of input), as determined by serial dilution.

Immunostaining and FACS® analysis of PMN antigen expression

To measure the expression of selected surface antigens, 5 x 10^5 PMN were suspended in 40 μl RPMI-HSA containing purified cryptococcal antigens at the designated concentrations. Mixtures were incubated for 30 min. at 37^{o}C on a rotation wheel after

they had been heated for 3 min. in a water-bath at 37°C. Then 10 µl of RPMI-HSA containing 0.25 - 0.5 µg of FITC-conjugated mAbs directed against CD11b (IgG_1; ATCC 44a), CD18 (IgG_{2a}; BD, Mountain View, CA-USA), or CD62L (IgG_{2a}; Leu-8, BD) was added and the incubation was continued for 30 min. on ice. Heparinized or EDTA-treated whole blood samples were analyzed using an identical staining protocol with a final incubation step in Lysing solution (BD) to eliminate red cells. Neutrophils were distinguished from other leukocytes by differences in forward (FSC) and side scatters (SSC) in FACS® analyses.

Expression levels of chemoattractant and TNF receptors on PMN were measured using the same methodology. We determined the expression of IL8-R type A and PAF-R using mAbs to CDw128A of the IgG_{2b} subclass and to PAF-R (IgG), both from Alexis Corp. (San Diego, USA). Surface expression levels of C5a-R and fMLP-R were determined using an anti CD88 mAb (IgG_{2a}, Serotec, Oxford, UK) and anti-fMLP-R mAb (IgG_1, PharMingen/BD), respectively. Since these different mAbs were not FITC-labeled, binding was visualized by incubation with FITC-conjugated goat F(ab') anti-mouse IgG (DAKO, Carpinteria, CA USA) as a secondary reagent to recognize the mAbs. To quantify the surface expression of the two receptors for TNF (p55 and p75) we used FITC-labeled antibodies of IgG_1 and -$_{2a}$ subclasses, respectively, which were purchased from R&D Systems (Minneapolis, MN USA). Isotype-matched conjugated mAbs (mouse IgG_1-FITC and mouse $IgG_{2a/b}$-RPE) not specifically reacting with PMN were purchased from DAKO and used as controls to exclude non-specific or Fc-related binding. The labeled cells were washed twice and analyzed with a FACS® or incubated with the secondary antibodies prior to analysis.

Recording of cytosolic Ca^{2+} concentration

To measure chemoattractant-induced Ca^{2+} fluxes, PMN were loaded with 2 µM Fluo-3-AM in RPMI/HSA for 15 min. at 37°C under agitation, washed with buffer and suspended at 10^6/ml in RPMI/HSA. The fluorescence (530 nm) of each 0.5-ml sample was measured to determine the basal Ca^{2+} level. Since Ca^{2+} fluxes are very rapid and transient, 5 µl reagent was added under vortexing and the sample was analyzed immediately by FACS®. For each sample nine measurements (0 through 8) of 2,000 cells were performed, each requiring an average time of ten seconds for sampling and data saving before the next acquisition was started. Samples were analyzed after gating the PMN population, thereby excluding cell debris and nonspecific staining.

TNF-R ELISA

Soluble TNF-R p55 and p75 shed from the surface of PMN were measured using a commercially available human ELISA kit (R & D Systems Europe, Abingdon, UK) according to the manufacturer's protocol. The minimum detectable doses of sTNF-R for these assays were typically less than 3 pg/ml for p55 or 1 pg/ml for p75. All data are expressed as means ± SEM.

Generation of antibodies directed against MP-4 and immunodetection

A rabbit antiserum directed against MP-4 was prepared by primary subcutaneous injection of two New Zealand White rabbits with 100 μg of MP-4 in complete Freund's adjuvant followed by a booster of 50 μg MP-4 in incomplete FA. The serum specifically recognized MP-4 migrating between 25 and 28 kDa, as shown (Figure 5 panel A) by standard Western blotting and ECL detection (Amersham Pharmacia Biotech, Uppsala, Sweden). When indicated, samples were pre-adsorbed to rabbit IgG purified from pre-immune serum using a protein G Sepharose 4 Fast Flow column (Amersham Pharmacia Biotech). Shortly, isolated pre-immune IgG was coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) beads. Immobilized IgG was added to the patient serum and CSpF samples and non-specific IgG-antigen interactions were allowed to occur for 30 min. at room temperature (batch binding).

RESULTS

Effects of cryptococcal capsular antigens on PMN migration toward IL-8, PAF and fMLP

IL-8, fMLP and PAF are examples of inflammatory mediators causing neutropenia because of rapid sequestration of PMN within the post-capillary venules of target organs. We evaluated the relative potency of capsular antigens to inhibit the migration of PMN toward these chemoattractants. Therefore, we first purified GalXM and three different MP from ΔCneF. Next, we confirmed that PMN were able to migrate toward IL-8, PAF, or fMLP in a dose-dependent manner (not shown). In our initial experiments we obtained almost identical results with CneF and ΔCneF, which was unexpected since GXM is considered to be the antigen exclusively responsible for the inhibition of PMN migration into the CSpF. We were able to detect a statistically significant inhibition of migration by adding ΔCneF to PMN migrating toward fMLP (18% inhibition, P=0.002) or IL-8 (23% inhibition, P=0.025; Figure 1A). We then added PMN incubated with MP-4 or other capsular antigens at the indicated optimal concentrations to the upper chamber and compared the inhibitory effect on PMN migration for each of them. GalXM (89%), GXM (46%) and MP-4 (98%) all caused significant ($P<0.01$) inhibition of migration toward IL-8, with MP-4 being the most potent (Figure 1A). GXM, GalXM and MP-4 all impaired migration toward fMLP, albeit to a somewhat lesser extent, with migration being inhibited by 66% (P=0.05), 60% (P=0.006) and 56% (P=0.0004), respectively. Migration toward PAF was completely blocked by MP-4 (P=0.015) and significantly inhibited by MP-2 (51%, P=0.007), GalXM (28%, P=0.0003) and GXM 70% (P=0.004). Because this inhibition could be caused by down-regulation of the receptors for these chemoattractants by MP-4, we analyzed the expression of the receptors for IL-8, fMLP, and PAF on PMN. There were no significant changes in the expression of

these receptors, although the number of fMLP and PAF receptors was slightly increased and the number of IL-8 receptors slightly decreased (not shown). Addition of polymyxin B to exclude any possible influence of trace amounts of LPS yielded identical results.

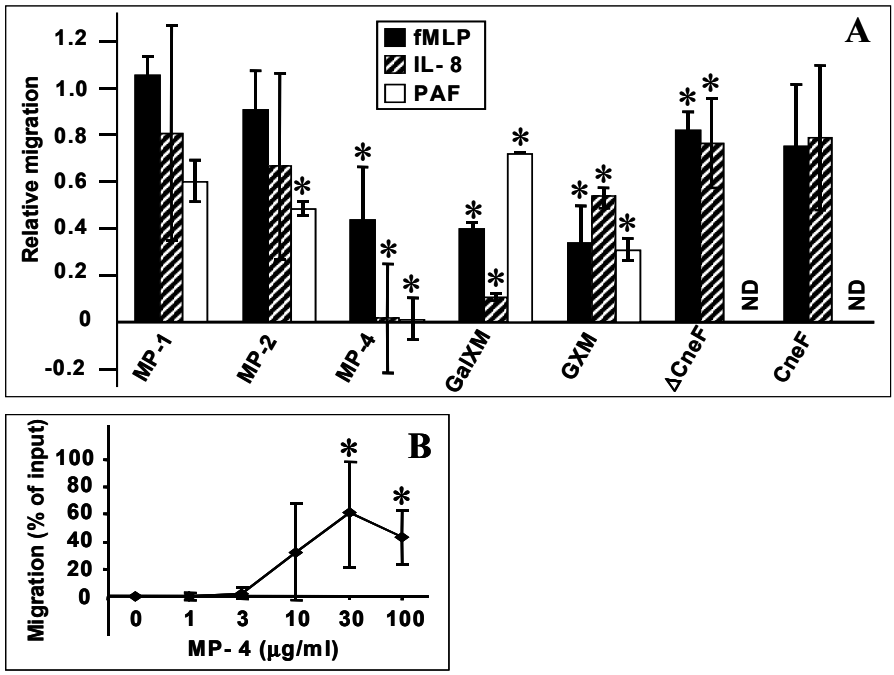

FIGURE 1. Cryptococcal capsular antigens affect PMN migration.
A. Inhibition of PMN migration toward chemoattractants. BCECF-AM-labeled PMN in the presence or absence of fungal antigens were added to the upper chamber of a transwell device. The migration of cells toward chemoattractants added to the lower chamber was quantified after 1 hour. MP were added to a concentration of 100 µg/ml, at which inhibition reached a plateau. GXM and GalXM were present at a final concentration of 1 mg/ml. Y-axis values represent migration toward chemoattractants in the presence of the indicated antigens relative to migration in the presence of buffer only (value = 1). Data are the means ± SEM derived from 3 (PAF; 100 nM final concentration), 5 (fMLP; 10 nM final concentration) to 7 (IL-8; 10 nM final concentration) independent experiments, except for GXM and GalXM (n = 2). Statistically significant data (2-tailed Student's t-Test) are marked by asterisks. **B.** MP-4 has intrinsic chemotactic activity. A concentration series of MP-4 was added to the lower compartment. Values (± SEM; n = 6) represent migration as percentage of the total amount of PMN (value = 1) added to the upper chamber.

MP-4 has intrinsic chemotactic properties

To determine the nature of the potent blocking effect of MP-4 on PMN migration we assayed the intrinsic chemoattractive activity of MP-4. MP-4 attracted PMN in a concentration-dependent manner (Figure 1B). Maximum migration (61% of input; P=0.007) was reached at an MP-4 concentration of 30 µg/ml whereafter migration started decreasing, probably due to saturating diffusion of MP-4 into the upper chamber. This observation was confirmed by the conventional under-agarose assay (32) for detection of chemotaxis (not shown). In this assay, the potency of MP-4 at 200 µg/ml equaled that of the positive control (PHS). Chemotaxis toward GXM (1

mg/ml) could not be detected with this less-sensitive assay. Importantly, the under-agarose assay permits measurement of both chemotaxis and spontaneous migration. The directed migration toward MP-4 observed in this assay, demonstrates that MP-4 is truly a chemoattractant and not simply acting as a chemokinetic agent. Furthermore, when 0.1 ng to 10 μg LPS/ml was added to the medium, in both assays no migration of PMN was observed, thus ruling out the possibility that the effects of MP-4 were caused by LPS contamination (not shown).

Ca^{2+} mobilization data indicate cross-desensitization of chemoattractant receptors by MP-4

The above results indicated that MP-4 might exert its effect by acting as a chemoattractant or by mimicking the action of chemoattractants. We therefore determined whether MP-4 could trigger an intracellular Ca^{2+} signal as occurs when chemoattractants bind to their G-protein coupled receptors. Figure 2A shows a steep increase in $[Ca^{2+}]_i$ in Fluo-3-AM-loaded PMN following stimulation by MP-4.

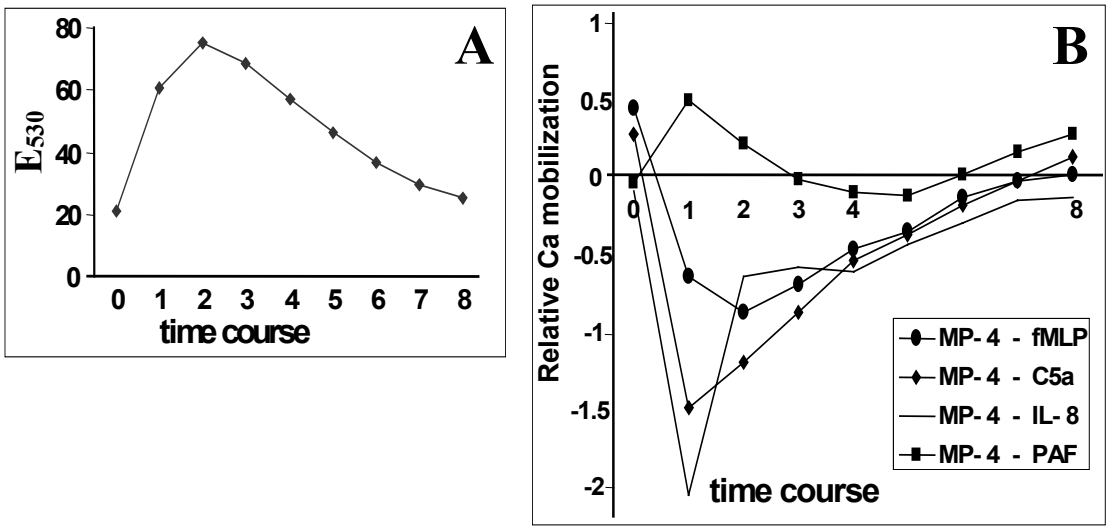

FIGURE 2. Interference of MP-4 with chemoattractant-induced intracellular Ca^{2+} mobilization. **A.** MP-4 transiently increases $[Ca^{2+}]_i$ in human PMN. Fluo-3-AM-loaded PMN were stimulated with MP-4 (10 μg/ml) and immediately analyzed for the induction of Ca^{2+}-fluxes. Y-axis represents fluorescence values (E_{530} nm). Nine subsequent measurements (0 through 8) were performed (X-axis); time point zero refers to the non-stimulated control. Each time point required an average time of ten seconds for sampling and data saving before the next acquisition was started. **B.** Desensitization by MP-4 of Ca^{2+} mobilization induced by fMLP, C5a and IL-8, but not PAF. Duplicate samples of PMN were incubated with MP-4, after which one tube was immediately used for Ca^{2+}-flux measurements. Once fluorescence decreased to baseline levels fMLP (10 nM), C5a (200 nM), IL-8 (10 nM), or PAF (100 nM) was added to the parallel tube and responses were recorded. Depicted is the Ca^{2+} mobilization elicited by the indicated chemoattractants after pre-incubation with MP-4 relative to the Ca^{2+} pattern obtained after chemoattractant stimulation alone, calculated by the following equitation: Relative Ca^{2+} mobilization = (response value after preincubation–direct response value)/ response value after preincubation. Depicted data are from one representative experiment of at least 3 independent experiments with qualitatively similar results.

Activation of chemoattractant receptors can result in homologous and heterologous desensitization, i.e. down-regulation of cellular responses after a second challenge with a G-protein coupled receptor ligand (33). In desensitization experiments, pretreatment of PMN with MP-4 almost completely prevented the IL-8-, fMLP- or C5a-, but not PAF-induced Ca^{2+} flux (Figure 2B). Inversely, pretreatment of PMN with the mentioned chemoattractants abrogated the Ca^{2+} flux induced by MP-4. These results suggest that MP-4 exerts its anti-inflammatory activity by interfering with chemoattractant-receptor signal transduction pathways.

Effects of cryptococcal antigens on the expression of CD11b/18 and L-selectin in the presence or absence of serum and bivalent cations

Stimulation of leukocytes with different chemoattractants such as fMLP, C5a and LTB4 causes a rapid decrease in the number of surface CD62L molecules and a concomitant inverse regulation of Mac-1/CR3 (27). Given the observed functional resemblance of MP-4 and other chemoattractants, we investigated whether MP-4 resembles these true-type chemoattractants in this respect. Culture of isolated PMN with purified MP-4 at 2 μg/10^6 cells resulted (Figure 3C-D) in a significant decrease in the amount of cell-surface CD62L (68%; P=6E-5) and in up-regulation of surface Mac-1 (2.2-fold; P=7E-4). MP-4 was more potent than GXM or CneF in evoking this response (Figure 3A-B). The MP-4 response almost equaled that induced by fMLP, which caused a decrease in surface CD62L of 74% and an almost 3-fold increase in Mac-1 expression. ΔCneF caused a significant down-regulation of CD62L (47%, P=4E-9) and up-regulation of CD11b (64%, P=9E-8). Neither GXM, GalXM (not shown), MP-1 (not shown), MP-2 (not shown), nor CneF induced significant CD62L loss or CD11b gain in the absence of serum. These data provide evidence that MP-4 is the capsular component that has the greatest effect on the expression of CD62L and Mac-1.

In order to study the effect of MP-4 on PMN under physiological conditions, we incubated heparinized blood with purified MP-4 and compared the observed effects to those obtained with CneF, ΔCneF, and GXM. Under these circumstances, ΔCneF (75%; P=0.02), GXM (149%; P=0.02), and MP-4 (195%; P=0.008) significantly increased the expression of CD11b (Figure 3A and C). The relative values for CD18 (not shown) were similar to those observed for CD11b. In addition, GXM (37%; P=0.02) and MP-4 (43%; P=2E-4) induced a significant loss of CD62L (Figure 3B and D). However, both have been reported to activate the complement cascade in serum to generate C5a, which then can induce the observed effects on the expression of surface molecules (24;29). To investigate the contribution of complement activation to MP-4 activity, we studied the regulation of CD62L and Mac-1 expression in the absence of bivalent cations by using EDTA-treated blood. Apart from fMLP, which was used as positive control, MP-4 was the only component able

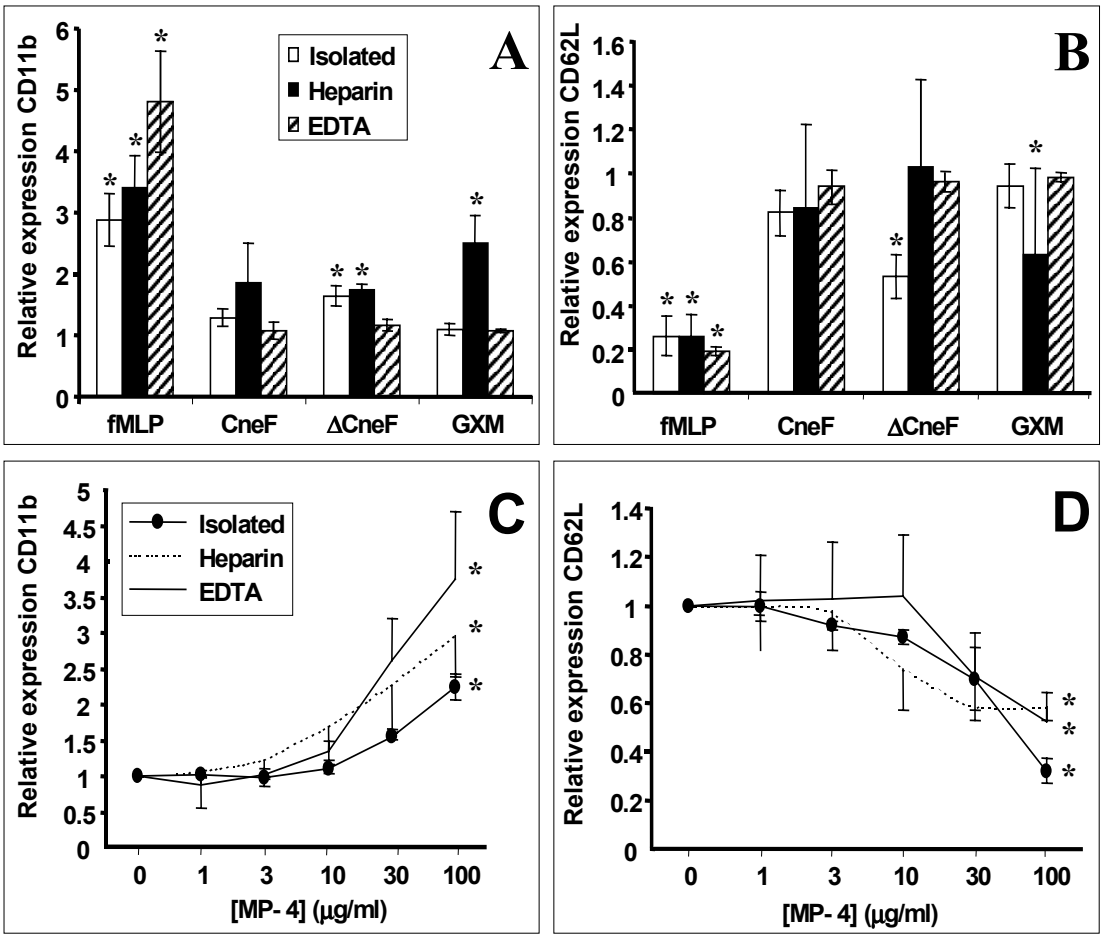

FIGURE 3. Cryptococcal capsular antigens induce CD62L shedding and up-regulation of CR3 expression on human PMN by a mechanism not requiring serum or bivalent cations.
The surface expression of CD62L and CR3 of PMN either isolated as described, or present in heparinized or EDTA-treated blood was compared. **A, B.** Bars indicate the expression (± SEM) of L-selectin and CD11b after incubation of PMN with fMLP (10 nM), GXM (1 mg/ml), CneF, or ΔCneF relative to the expression after incubation with RPMI/HSA (value = 1). **C, D.** Lines represent the level of expression of the indicated surface molecules after incubation with MP-4 at 0, 1, 3, 10, 30, or 100 µg/ml. Changes in the expression of CD18 were similar to those measured for CD11b. For clarity reasons, expression values in heparinized blood are depicted - SEM, values in EDTA blood are depicted + SEM and values of isolated PMN ± SEM. Statistically significant data (2-tailed Student's t-Test) are marked by asterisks.

to alter the expression of CD11b or CD62L significantly under these circumstances (Figure 3). MP-4 dose-dependently increased the expression of CD11b to a maximum of 3.8-fold (P=0.015; Figure 3C) and decreased surface CD62L expression by 48% (P=0.005; Figure 3D). The same data for GXM were 7% and 2%, respectively. To exclude any donor-specific influence, isolated PMN, heparinized blood and EDTA-treated blood were collected from the same donors. For heparinized blood and PMN absolute fluorescence values were in the same range. EDTA-treated blood showed an almost 2-fold reduction in basal CD11b/CD18 expression, whereas CD62L levels were 2-fold higher. These observations demonstrate that MP-4 affects PMN function by a mechanism not requiring serum proteins nor extracellular Ca^{2+} or Mg^{2+}.

MP-4 induces shedding of TNF-R from the surface of PMN

Results showed that MP-4 desensitized PMN, which explains the inhibited PMN influx into the brains of patients with meningitis. In conflict with this clinical observation is the induction of TNF-α expression observed *in vitro* (10-13) because TNF-α is generally assumed to promote extravasation. Since at least two reports (34;35) demonstrated a substantial reduction in PMN influx at the site of infection after neutralization of TNF, we investigated the possible involvement of TNF scavenging in reducing CSpF leukocyte counts. To examine the possible role of TNF-R in cryptococcal meningitis, we measured the influence of capsular antigens on the generation of soluble TNF-R for the two types of TNF-R expressed on the surface of PMN: p55 and p75 (36). With a soluble TNF-R ELISA kit, the medium from freshly isolated PMN cultured for 1 h at 37°C contained clearly detectable levels of TNF-R (108 pg/ml and 186 pg/ml p55 and p75, respectively), suggesting that cell surface

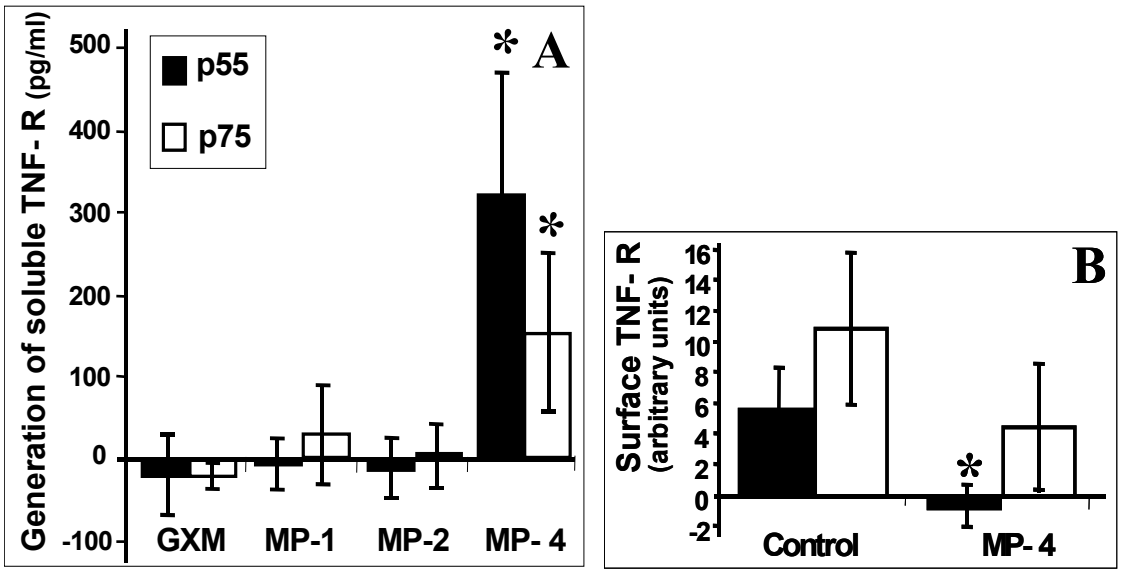

FIGURE 4. Effects of cryptococcal capsular antigens on the expression of TNF-α receptors on PMN: shedding causes down-regulation.
(A) Quantification of soluble TNF-R levels in supernatants (20 µl) of PMN (4.10^5) by ELISA. Data are concentrations of soluble TNF measured after incubation of PMN with GXM (0.5 mg/ml) or MP (50 µg/ml) for 1 h at 37°C minus the matched control value (PMN from the same donor incubated with PBS alone) and represent four independent experiments with blood from different donors and ELISA performed in duplicate. **(B)** Effect of MP-4 on the PMN surface expression of TNF-α receptors. Bars indicate the expression (± SEM) of TNF-R p55 and p75 after incubation of PMN with MP-4 (30 µg/ml) or RPMI/HSA (control) in arbitrary fluorescence units. Statistically significant data (2-tailed Student's t-Test) are marked by asterisks.

proteolysis is part of the normal turnover of TNF-R. Stimulation of PMN with MP-4 resulted in a significant increase of both sTNF-R p55 (4-fold; P = 0.0002) and p75 (1.8-fold; P = 0.002) in the supernatants (Figure 4A). None of the other tested capsular components had an effect on TNF-R shedding. Since soluble TNF-R

compete with TNF-α at the cellular receptor, we also measured the density of p55 and p75 on PMN after treatment with MP-4. We observed a significant (P = 0.01) down-regulation of surface p55 on PMN after addition of MP-4. p75 was also down-regulated however due to the large variability between individual donors this was not significant (Figure 4B).

MP-4 levels in Cryptococcosis patients

Using a rabbit polyclonal antiserum directed against MP-4, we measured the levels of MP-4 present in the serum and CSpF of patients suffering from cryptococcal meningitis by standard Western blot analysis.

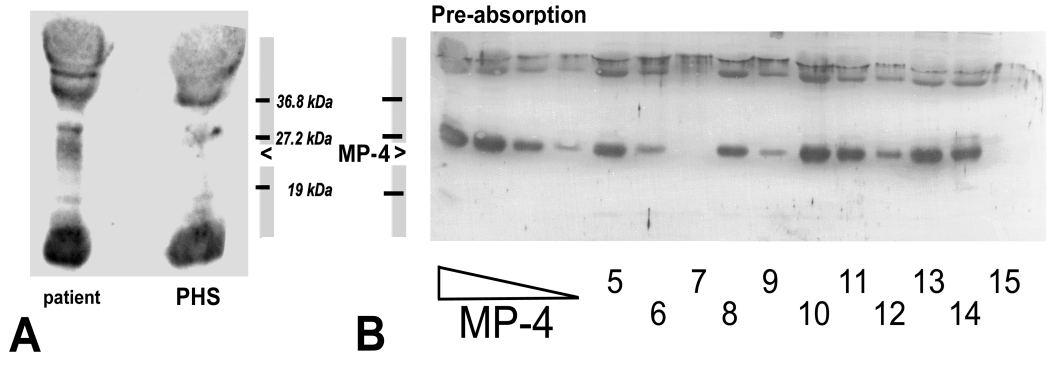

FIGURE 5. Detection of MP-4 in the serum and CSpF of patients suffering from cryptococcal meningitis. *Immunoblotting*. **(A).** Comparison of the amount of MP-4 detectable in serum collected from a cryptococcosis patient and pooled human sera; separation by SDS-PAGE on a 15% PA gel. **(B).** Quantification of MP-4 in the serum and CSpF of patients suffering from cryptococcal meningitis. Lanes 1-4 contain 7.5 µl of purified MP-4 in a concentration of 30, 10, 3 and 1 µg/ml, respectively, resolved by SDS-PAGE on a 20% gel. In lanes 5 to 14 - which were all pre-adsorbed to pre-immune IgG - we added matched CSpF (7.5 µl initial volume; lanes 5-9) and serum (1.5 µl; lanes 10-14) samples. Lane 15 contains PHS (1.5 µl initial volume). Patient samples are from HIV-infected cryptococcosis patients (5-8; 10-13) and from a non-HIV-infected patient (9; 14). Proteins were electroblotted to nitrocellulose membranes and incubated with the described primary antiserum in a 1:100 dilution. Filters were subsequently incubated with goat ant-rabbit IgG linked to HRP. Bound HRP was visualized by ECL. Arrowheads indicate the position of MP-4.

In panel A, we analyzed and compared the amount of MP-4 detectable in serum collected from a cryptococcosis patient and pooled human sera. Although the non-adsorbed antiserum directed against MP-4 is cross-reactive to several serum proteins we clearly detected a specific band at the putative molecular weight of MP-4 in patient- but not pooled serum. The identity of this putative MP-4 band was confirmed in panel B lanes 1 to 4 where we applied purified MP-4 in a concentration of 30, 10, 3 and 1 µg/ml, respectively, as a reference. In this panel, all samples were pre-adsorbed to pre-immune IgG, which removed all cross-reactive proteins except for one doublet at a high molecular weight position. In lanes 5-14 we analyzed and compared the

amounts of MP-4 detectable in paired samples of CSpF and serum collected from HIV-infected cryptococcosis patients (5-8, 10-13) or a non-HIV-infected patient (9 and 14). MP-4 is clearly detected in the serum of these patients at a level strongly exceeding that present in CSpF, providing evidence for the formation of a gradient of MP-4 over the blood-brain barrier. The amount of MP-4 present in the serum of these patients was calculated relative to the standards after densitometric scanning of the blots and ranged between 10 and 55 μg/ml, respectively, by densitometric scanning of the autoradiograph. The concentration present in CSpF varied between < 1 μg/ml and 7 μg/ml.

DISCUSSION

The potency of *C. neoformans* culture supernatants to inhibit leukocyte migration was recognized almost half a century ago (37). Using purified cryptococcal antigens we now show that MP-4 is the most active capsular antigen in inhibiting migration toward chemoattractants. Moreover, we provide direct evidence for the presence of MP-4 in the serum of patients with disseminated cryptococcosis. Below we discuss the role of these and other recent findings in the pathogenesis of the disease and focus on the mechanism of action of MP-4.

MP-4 strongly inhibited PMN migration toward fMLP, PAF and IL-8 (Figure 1A). Conversely, MP-4 was found to be a potent chemoattractant *in vitro* (Figure 1B). The paradox emerging here can be explained by a phenomenon called cross-desensitization of chemoattractant receptors, as initially described by Sabroe *et al* (33). This hypothesis was confirmed by our observation that pre-incubation of PMN with MP-4 impaired Ca^{2+} mobilization by fMLP, IL-8 and C5a - but not PAF (Figure 2B). Although speculative at present, this difference in the regulation of chemoattractant-mediated responses is likely to be related to G-protein usage, as suggested by the distinct pertussis toxin sensitivity of the receptors for IL-8, fMLP, and C5a versus PAF, with the former being sensitive and the latter at least partially resistant (38;39).

The pathophysiological role of MP-4 is thus to functionally mimic the action of chemoattractants, resulting in a premature activation of PMN. Such a role for MP-4 is consistent with the observed loss of CD62L and concomitant up-regulation of CD11b/18 (Figure 3), because chemoattractants affect the expression of these molecules (27;40;41). Kishimoto *et al.* showed that PMN shed CD62L from the cell surface within minutes after activation with chemotactic factors. Both the expression and activity of CD11b/18 were greatly increased (27). This work founded the current view that by the time chemoattractants arrive in the circulation, CD62L-mediated neutrophil rolling has already taken place and the integrin-mediated attachment takes over. In addition, Luscinskas *et al*. showed that IL-8, fMLP and C5a, besides

inhibiting the attachment of PMN to cytokine-activated endothelial monolayers, promoted the rapid detachment of tightly adherent PMN from activated endothelial cells, and abolished transendothelial migration (40). Finally, in IL-8 transgenic mice, but not in non-transgenic littermates, PMN migration into the inflamed peritoneal cavity was severely inhibited (41). Thus, although fMLP, IL-8, and C5a have been characterized primarily as chemoattractants, they can exert a wide range of modulatory effects on PMN-endothelial adhesive interactions.

The association between MP-4 and down-regulation by shedding of surface TNF-R (Figure 4) is also in agreement with the proposed role of MP-4 as chemoattractant. Several *in vitro* studies (42;43) demonstrated that within minutes of chemoattractant incubation both TNF-R p55 and p75 were down-regulated from the surface of PMN. At the same time, soluble TNF-R appeared in supernatants, in amounts proportional to the extent of down-regulation. This suggests that shedding is the major mechanism leading to the loss of p55 and p75 upon chemoattractant activation. Our demonstration of increased amounts of shed receptors in conditioned media of neutrophils exposed to MP-4 supports this hypothesis. *In vivo*, scavenging of TNF-α by soluble TNF-R is likely to contribute to the observed lack of leukocytes in the CSpF of patients for at least two reasons. First, others and we have shown that cryptococcal antigens stimulate the production of TNF-α by leukocytes in the presence of serum (12;13). Second, TNF-α is known to play a crucial role in the control of organ infiltration. TNF-α activates the endothelium to cause leukocyte adherence (4;44) and triggers leukocyte infiltration of lung, liver, and kidney in control mice but not p55-deficient mice (44). Finally, TNF-α can cause profound systemic vasodilatation and subsequent hypotension (16), allowing CD62L-mediated adherence of leukocytes to the vessel wall (17-19). Together, these *in vivo* data indicate that the presence of TNF-α positively correlates with leukocyte organ infiltration.

In a series of studies by Dong and Murphy and our group, GXM has been shown to possess anti-inflammatory properties that prevent PMN from accumulating in *C. neoformans* infected tissues (22-25;45). The present work shows that MP-4 shares most of these properties with GXM, but is more potent. Although analyzed before (22;24;45) no direct effects of MP on PMN migration have been reported. These conflicting results can be explained by differences in the purification strategy used here. Previously, MP were eluted from the Con A column using a 0.2 M α-methyl-D-mannopyranoside (α-mDm) step elution protocol. Recently, however, we reported that MP-4 elutes from this column at 0.4 M α-mDm (30), thus indicating that MP-4 was never present in the previously used MP preparations. Since analysis of MP-4 reveals a signal eluting between polysaccharide calibration standards of 18 and 26 kDa in gel filtration chromatography (not shown), this component also was not

present in ΔCneF used in the studies mentioned above, because the culture filtrates were concentrated using a 30 kDa cut-off cassette.

Our analysis of the concentrations of MP-4 present in the serum of patients with cryptococcal meningitis has revealed a concentration range matching that required to obtain the described *in vitro* effects - thereby confirming the biological significance of our data. The concentrations required are high when compared to serum concentrations for mannoproteins from other fungi but are in line with experiments - both *in vitro* and *in vivo* - performed with other mannoproteins from *C. neoformans* (46;47). The relatively high concentrations of MP-4 detected in 4 out 5 patients can be partly explained by the fact that these patients were all newly admitted patients for whom treatment had not been started at the moment of sampling. In order to further explain the relatively high concentrations required for cryptococcal mannoproteins, experiments are in progress to determine the biological activity and half-life of MP-4 *in vivo* as well as *in vitro*.

In summary, we have shown that MP-4 has potent anti-inflammatory activity that may be responsible for the poor cellular infiltration of PMN into the CSpF of cryptococcosis patients. Given our hypothesis that MP-4 exerts its action largely by its intrinsic chemoattractive properties, the balance between pro-inflammatory and anti-inflammatory effects will depend critically on the temporal and spatial distribution of MP-4. Interestingly, even in patients with cryptococcal meningitis MP-4 concentrations in serum strongly exceed the levels detected in CSpF. We hypothesize that this distribution might be due to active drainage of MP-4 from CSpF to blood, resulting in increasing blood concentrations of MP-4 in the course of the disease. Active drainage will prevent both the migration of PMN toward MP-4 present in the CSpF, as well as the proper response to extra-vascular chemoattractants because of the premature activation of PMN. Alternatively, MP-4 expression might be strongly up-regulated during cryptococcal trafficking in the vascular compartment, before the brain section is reached. Environmentally induced expression of capsule genes is not unprecedented. Several nutrients, e.g. iron and CO_2, have been demonstrated to affect the composition of the cryptococcal capsule (48). We are currently testing the ability of MP-4 to limit neutrophil infiltration in vivo using a rabbit model for BM.

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CHAPTER 9

General discussion

Cryptococcosis is the leading cause of life-threatening mycological disease of the central nervous system, with a high worldwide mortality and morbidity in immunocompromised patients (1). In the Netherlands from 1996 on incidence numbers are declining, in parallel with incidence numbers in the rest of the Western world (2). This decline is due to the introduction of highly active antiretroviral therapy (HAART), as described in **chapter 2**. In countries that can not afford costs of HAART, like for example in Sub Saharan Africa and India, cryptococcal related infection are an enormous problem (3;4), in Zimbabwe *C. neoformans* is the most important cause of meningitis in adults. Healthy subjects rarely become infected by *C. neoformans*. In the Dutch surveillance study of cryptococcosis patients, in only 7 patients out of 268 (2.6%) no underlying disease could be determined despite extensive work-up. Apparently, in healthy individuals host defense against cryptococci is very efficient, considering the ubiquitous presence of the yeast in nature. On the other hand in some immunocompetent patients the diagnose might remain undiagnosed.

C. neoformans enters the body by inhalation of poorly encapsulated yeast cells. In the lungs innate immune responses like phagocytosis of *C. neoformans* by resident macrophages (alveolar macrophages) take place. In **chapter 3** a new, rapid and objective method is described, to measure interaction between the yeast *C. neoformans* and adherent phagocytic cells. The major advantage compared to flow cytometry is that phagocytosis of adherent cells can be measured instead of cells in suspension and therefore, long term incubations can be studied. This way of studying this interaction probably resembles the *in vivo* situation more closely.

Phagocytosis and killing of *C. neoformans*, in the presence of complement, by neutrophils, monocytes, and macrophages is very effective (5-7). As described in **chapter 4**, in contrast to many other micro-organisms like *E. coli* (8), *M. tuberculosis* (9), and influenza A virus (10), pulmonary surfactant proteins do not support phagocytosis of *C. neoformans*. FITC-SP-A bound in a concentration-dependent manner to acapsular *C. neoformans*. This binding was inhibited by glucose and mannose, meaning that SP-A binding can occur via its carbohydrate-binding region, but does not result in enhanced phagocytosis. It is not known how *C. neoformans* escapes surfactant protein mediated phagocytosis, but this escape makes it easier for cryptococci to grow and synthesize its capsule in alveolar spaces.

Cytokines and chemokines

Cytokines that are present during the initiation of an immune response will determine whether CMI or B-cell development will take place (11). Pulmonary clearance of the encapsulated yeast *C. neoformans* is considered to be primarily controlled by CMI suggesting a predominantly type 1 cytokine response is essential. Type 1 cytokines include IFN-γ, IL-12, and TNF-α, whereas type 2 cytokines include IL-4, IL-5, IL-6,

IL-10, and IL-13 (11). In **chapter 5** the time kinetics of cytokine release in PBMC of healthy donors stimulated with cryptococcal capsular polysaccharides indicate an autocrine-paracrine network regulation of the cytokines. Proinflammatory cytokines like TNF-α and IFN-γ were released early while IL-1ß, IL-6 and IL-8 showed a more sustained release, and IL-10 was clearly increasing over time. We did not observe the tendency for a particular cryptococcal strain or capsular component to induce a type 1 or type 2 response, but in the first 3 hours a more type 1-like pattern was observed for all the different strains and components. This profile would facilitate an adequate cellular mediated immunity in normal healthy donors. Stimulation of PBMC for 18 h was associated with an increase in IL-10 levels, and a decline in TNF-α and IFN-γ release. This corresponds to a shift towards a more type 2 cytokine response, stimulating the induction of humoral immunity.

Cytokine induction in the host defense against *C. neoformans* might be a double-edged sword, playing beneficial or detrimental roles, especially in the setting of HIV infection. TNF-α for example, despite its pivotal role in initiating and amplifying anti-cryptococcal immune responses (12;13), has been described to accelerate HIV progression (14;15). On the other hand, IL-10 can down regulate HIV replication (16) but potentially dampens important protective immune responses against cryptococcal infection. This will have important implications for designing cytokine-based therapeutic strategies against cryptococcal infections in HIV-infected patients.

HIV-1 infection of macrophages increases mRNA levels of the chemokines MIP-1α, MIP-1β, and RANTES as described in **chapter 6**. Moreover, both HIV infection and *C. neoformans* or GXM stimulation of macrophages resulted in an additional enhancement of chemokine expression. MIP-1α, MIP-1β, and RANTES attract activated T cells, dendritic cells, and monocytes by binding to specific CC chemokine receptors (CCR) on these cells, including CCR5 (17). CCR5 also functions as a coreceptor for macrophage-tropic, nonsyncytium-inducing strains of HIV. MIP-1α, MIP-1β, and RANTES suppress replication of macrophage-tropic (but not lympho-tropic) HIV strains in vitro, presumably by competitive inhibition of HIV attachment to CCR5 (18;19). Previously, it was demonstrated that *C. neoformans* induce HIV replication in latently infected monocytic cell lines (20-22). In clinical studies, an increase in HIV viral load was observed in patients suffering from cryptococcosis (23). Our finding that HIV-infected macrophages, and Huangs finding that HIV-positive PBMC produce MIP-1α, MIP-1β, and RANTES would suggest that HIV viral load should decrease, rather than increase, during fungal infections. Two factors of this apparent paradox have been suggested (17). First, as HIV infection progresses to AIDS, there tends to be a change in corecptor usage. This results from macrophage-tropic HIV strains (which use CCR5) being replaced by lympho-trophic strains (which prefer CXCR4) (18;19). MIP-1α, MIP-1β, and RANTES bind CCR5 but not CXCR4. Moreover, for lympho-trophic strains, MIP-1α, MIP-1β and RANTES may

directly stimulate HIV replication (19). Thus, as cryptococcosis usually occurs in advanced HIV infection, release of MIP-1α, MIP-1β, and RANTES stimulated by *C. neoformans* increase HIV viral load rather than decrease it. Second, *C. neoformans* stimulate release of TNF-α and the nuclear translocation of the transcription factor NF-κB, both of which induce HIV replication (22;24).

Cryptococcal capsular polysaccharides

Chaka et al. found that, in contrast to bacterial meningitis, the local production of high levels of IL-8 is not associated with leukocyte infiltration allowing proliferation of yeast cells (25). The potency of *C. neoformans* culture supernatant to inhibit leukocyte migration was first described in 1956 (26). In a mouse model study, Dong and Murphy showed that the inhibition of PMN infiltration elicited by intravenous administration of the cryptococcal culture filtrate (CneF) is mainly due to GXM (27). Lipovsky et al. previously demonstrated that GXM significantly inhibited PMN migration toward IL-8 (28). Furthermore, it was shown that GXM delays translocation of leukocytes across the blood-brain barrier in a rabbit meningitis model (29). In **chapter 7** we have demonstrated a significant inverse correlation between the GXM ratio (serum/CSF) and the CSF leukocyte count in Dutch patients with cryptococcosis. These results could possibly be explained by the fact that GXM has been described to be a chemoattractant by itself (28). Second, a more specific interference with transendothelial migration of PMN could as well be expected. GXM sheds L-selectin from neutrophils (30), and can bind to CD18 adhesion molecules thereby blocking interaction of activated neutrophils with ligands on the endothelium (31). Thirdly, in patients in which cryptococcosis is suspected *C. neoformans* cultures and latex agglutination tests for cryptococcal antigen (GXM) in blood and CSF is performed. Antigen titers that were measured for diagnostic purposes were used in this study. There is a possibility that other capsular polysaccharides, like MP4, divided over the brain/blood compartment in the same ratio as GXM, is responsible for the observed effect, but this has not been measured until now.

Initially, aim of further study was to investigate the molecular mechanism by which GXM is able to prevent leukocyte migration toward chemoattractants. Surprisingly, we found that cryptococcal culture filtrate (CneF) from GXM-producing and genetically modified nonproducing (ΔCneF) strains, both prevented PMN migration towards PAF, IL-8 and fMLP (**chapter 8**). Using a rabbit polyclonal antiserum directed against MP-4, evidence was provided for the presence of MP-4 in serum and CSF of patients with disseminated cryptoccocosis. The paradox that MP-4 strongly inhibited PMN migration toward PAF, IL-8 and fMLP and that MP-4 was found to be a potent chemoattractant in vitro itself, can be explained by a phenomenon called cross-desentization of chemoattractant receptors. This hypothesis was confirmed by our observation that preincubation of PMN with MP-4 impaired Ca2+ mobilization by

IL-8, fMLP, and C5a, but not PAF. Thus, the pathophysiological role of MP-4 is to functionally mimic the action of chemoattractants, resulting in a premature activation of PMN. The association between MP-4 and downregulation by shedding of surface TNF-R is also in agreement with the proposed role of MP-4 as chemoattractant. Several studies demonstrated that within minutes of chemoattractant incubation, both TNF-R p55 and p75 were down-regulated from the surface of PMN. At the same time, soluble TNF-R appeared in supernatants, in amounts proportional to the extent of down-regulation. This suggests that shedding is the major mechanism leading to the loss of p55 and p75 upon chemomattractant activation (32;33). Our demonstration of increased amounts of receptors shed in conditioned medium of neutrophils exposed to MP-4 supports this hypothesis. Several studies provide evidence that TNF-α correlates positively with leukocyte organ infiltration (34;35). In vivo, scavenging of TNF-α by soluble TNF-R is likely to contribute to the observed lack of leucocytes in the CSF of patients.

Future research

Neutrophil infiltration into the central nervous system is a dual-edged sword in that it protects the tissue from infection and injury but can also be detrimental to the host. In diseases such as bacterial meningitis or cerebrovascular ischemia there is evidence that a high influx of PMN plays an adverse role in the pathogenesis of neurological damage (36;37). In bacterial meningitis, the phagocytic capacity of PMN in CSF is often insufficient and the harmful effects of their cytotoxic products may outweigh their beneficial effects (38). Accordingly, it is now assumed that adjunctive therapeutic strategies with anti-inflammatory agents may favor neurological recovery (39-41). Therefore we will investigate the possibilities to use GXM and MP4 therapeutically.

At least two capsular components of the fungus *C. neoformans* have been identified to interfere with neutrophil migration into the CSF. Initially only GXM was used as a therapeutic agent to reduce the influx of PMN into the CSF during pneumococcal meningitis in a rabbit model (29). In GXM treated rabbits, there was a substantial delay in CSF total leukocyte count and percentage of PMN at 6h, as compared to non-treated animals. The histopathology of the brain at 8h demonstrated less inflammation and less margination of PMN in the GXM treated animals. Furthermore, the effects of GXM on both neutrophils and endothelium using a static adhesion models were studied (42). Pretreating PMN with GXM inhibited neutrophil adhesion to TNF-α-stimulated endothelium up to 44%. Treatment of TNF-α-stimulated endothelium with GXM led to a 27% decrease in PMN adhesion. Based on the findings that no additive inhibition of PMN adhesion was found when blocking mAbs against E-selectin were added after GXM pretreatment of PMN while these mAbs did decrease adhesion in the absence of GXM, GXM most likely exerts its effect on PMN by interfering with

E-selectin mediated binding. The use of blocking antibodies also showed that the inhibitive effect found after GXM treatment of endothelium probably involves interference with both ICAM-1 and E-selectin binding.

A second capsular component, MP-4 was identified as a potent inhibitor of neutrophil migration. For future research, further chemical identification, and methods to obtain sufficient amounts of MP-4 will have to be developed. For example, the expression of the protein part of MP-4 in an *E. coli* expression and purification system would upscale MP-4 production enormously. This will create the possibility to test MP-4 as an anti-inflammatory agent in for example a pneumococal meningitis model in rabbits, or in an ischemia/reperfusion damage model in rats. Future research will have to include the search to a possible MP-4 receptor on PMN. The effect of MP-4 on the expression of adhesion molecules on endothelial cells like ICAM-1, P-selectin and VCAM will provide insight in additional mechanisms by which MP-4 interferes with neutrophil migration.

Developing MP-4 as a possible therapeutic agent will raise questions of unwanted side effects. As described in chapter 5, MP-4 does stimulate PBMC of healthy donors to produce TNF-α, IFN-γ, IL-1ß, IL-6 and IL-8. These experiments were performed in the presence of 10% normal human serum, which resembles the situation *in vivo*. Part of the observed cytokine release by the different components of *C. neoformans* might be caused by indirect effects. Cytokine induction in PBMC by GXM depends on the presence of an intact complement system, whereas for GalXM and MP this process is partially facilitated by human mannose binding lectin, a heat stable component (43;44). The observed cytokine profile might be strongly influenced by the autocrine-paracrine cytokine network, since the presence of e.g. high levels of TNF-α induces IL-10 independent of the type of stimulus (45).

Be that as it may, MP-4 is able to activate PBMC in various ways. These activation mechanisms could possibly interfere with the application of MP-4 as an anti-inflammatory drug. However, the question remains how relevant the *in vitro* observations are for the *in vivo* situation. For example, in healthy non-anesthetized rabbits, intravenous administration of GXM was well tolerated (29). GXM is, like MP-4, able to activate PBMC in vitro (**chapter 5**). Furthermore, treating animals with GXM resulted in a reduction of the TNF-α peak levels in the CSF at 2 h after intracesternal inoculation of heat-killed pneumococci. Testing MP-4 in an animal model will be necessary to find out if activating properties of MP-4 are present and clinically important.

Further identification of MP-4 might reveal different parts of the molecule, which are responsible for different biological activities. Identification of the part specifically interfering with neutrophil migration will provide a safe and powerful anti-inflammatory agent.

This thesis describes several aspects of the human immune response against *C. neoformans* on its route from inhalation from the environment to the central nervous system. Studying the mechanisms by which cryptococci defend themselves against the human host, revealed components of *C. neoformans* which are able to inhibit neutrophil migration. The use of these defense mechanisms from cryptococci, provides possibly a new anti-inflammatory drug.

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Nederlandse samenvatting

Cryptococcose is een ziekte die wordt veroorzaakt door de gekapselde gist *Cryptococcus neoformans*. Bij patiënten met verminderde afweer is het een van de belangrijkste veroorzakers van ernstige infecties van het centrale zenuwstelsel, zoals hersenvliesontsteking. De afweer van een patiënt kan verminderd zijn door infectie met het HIV virus, gebruik van immunosuppressieve medicatie, of door het hebben van een hematologische maligniteit. In Nederland komt cryptococcose sinds 1996 steeds minder voor. In **hoofdstuk 2** beschrijven we dat dit wordt veroorzaakt door de introductie van krachtige antivirale therapie (highly active antiretroviral therapy, HAART) voor de behandeling van HIV. In landen die de hoge kosten voor HAART niet kunnen betalen, bijvoorbeeld in Afrika en India, is cryptococcose een enorm groot probleem.

Gezonde mensen raken nauwelijks geïnfecteerd met *C. neoformans*. In de studie die in hoofdstuk 2 wordt beschreven hebben we naar (bijna) alle Nederlandse patiënten met cryptococcose gekeken van 1986 tot 2000. In slechts 7 van de 268 patiënten (2,6%) konden we geen onderliggende ziekte vinden, ondanks uitgebreid aanvullend onderzoek. Blijkbaar is bij gezonde mensen de afweer tegen cryptococcen erg efficiënt zeker als je in overweging neemt dat cryptococcen veelvuldig in de natuur voorkomen.

C. neoformans komt het menselijk lichaam binnen door het inhaleren van dun gekapselde gist cellen. In de long komt de aspecifieke afweer op gang, zoals fagocytose (‘opeten’) van *C. neoformans* door de daar aanwezige macrofagen (alveolaire macrofagen). In **hoofdstuk 3** beschrijven we een nieuwe, snelle en objectieve methode om de interactie tussen de cryptococ en vastgehechte fagocyten te meten. Het grootste voordeel ten opzichte van een andere veel gebruikte methode om fagocytose te meten (flow cytometrie) is, dat de cellen naast elkaar vastgehecht zitten op de bodem van een plaatje in plaats van cellen in een reageerbuisje in oplossing, en dit biedt de mogelijkheid om gedurende langere tijd fagocytose te bestuderen. Deze situatie bootst de situatie in het menselijk lichaam beter na dan metingen in oplossing.

De aanval tegen binnengedrongen cryptococcen door het menselijk afweersysteem, in de aanwezigheid van complement (het complement systeem bestaat uit factoren in het bloed die in staat zijn om lichaamsvreemde indringers te herkennen en kapot te maken), door witte bloed cellen zoals neutrofiele granulocyten, monocyten en macrofagen is heel efficiënt. Zoals beschreven in **hoofdstuk 4** onttrekken cryptococcen zich aan fagocytose gefaciliteerd door de long surfactant eiwitten. Dit in tegenstelling tot vele andere micro-organismen zoals *E. coli, M. tuberculosis* en het influenza A virus. FITC-SP-A (een van de long surfactant eiwitten gelabeld met een groene kleurstof) bindt concentratie-afhankelijk aan niet gekapselde cryptococcen. Deze binding werd geremd door glucose en mannose, wat betekent dat SP-A binding plaatsvindt via het koolhydraat-bindende deel van het molecuul, maar dit resulteert

niet in versterkte fagocytose. Het is onbekend hoe de cryptococ zich aan de surfactant gemedieerde fagocytose onttrekt, maar door deze ontsnapping zal het makkelijker zijn voor de gist om te groeien en zijn kapsel aan te maken in de alveolaire ruimtes in de long.

Als de cryptococ zich eenmaal in de long heeft genesteld kan deze zich verder door het lichaam verspreiden. In het bloed zal de cryptococ in aanraking komen met witte bloedcellen, zoals monocyten. Dit proces leidt tot de productie van signaal moleculen, cytokinen genaamd. Cytokinen zijn een groep van serumeiwitten die zowel een beschermende als een ziekte modulerende rol kunnen spelen. Hele cryptococcen en gezuiverde kapsel componenten induceren in monocyten een vergelijkbaar cytokinenspectrum (**hoofdstuk 5**). Wel verschilde de kinetiek van de bestudeerde cytokines op de verschillende tijdpunten (3 en 18 uur). Een belangrijke suggestie voortkomend uit deze studie is dat gedurende het acute contact tussen het afweersysteem en cryptococcen een cytokine-spectrum wordt geïnduceerd dat gunstig is voor de opwekking van de cellulaire afweer en dat gedurende een langduriger contact het geïnduceerde cytokine spectrum zorgt voor een afbouw van de cellulaire immuniteit, ons belangrijkste afweermechanisme tegen *C. neoformans*.

Vanuit de bloedbaan gaan cryptococcen op weg naar de hersenen. Eenmaal in de hersenen aangekomen zijn de gisten relatief onbereikbaar voor het afweersysteem en kunnen ze zich vermenigvuldigen en zo grote schade aan het brein toebrengen. Waarom de cellen van het afweer systeem de met cryptococcen geïnfecteerde hersenen niet kunnen bereiken is niet goed bekend. Voor het migreren vanuit de bloedbaan naar de plaats van de cryptococcen infectie hebben witte bloedcellen een signaal nodig zodat ze weten waar de indringers zich bevinden. Dit signaal bestaat uit stoffen die ter plekke vrijkomen zodra de cryptococ en het afweersysteem samenkomen. Een groep van deze signaal stoffen heet chemokinen. Chemokinen trekken witte bloedcellen aan zodra ze herkend worden als alarmsignaal door receptoren op het oppervlak van witte bloedcellen. Zodra de witte bloedcellen het alarmsignaal op hun receptor opvangen worden ze actief en migreren ze vanuit de bloedbaan naar de plaats waar de chemokinen vandaan komen. MIP-1α, MIP-1β, en RANTES zijn chemokinen die voornamelijk monocyten aantrekken. In **hoofdstuk 6** hebben we bestudeerd of deze signaalstoffen daadwerkelijk vrijkomen als er contact is tussen cryptococcen en al dan niet met HIV geïnfecteerde macrofagen. HIV-1 infectie zelf zorgt voor een verhoging van de bestudeerde chemokinen MIP-1α, MIP-1β, en RANTES. Daarnaast werd een additionele verhoging van deze chemokinen gevonden als de macrofagen tevens werden gestimuleerd met *C. neoformans* of een van de gezuiverde kapselbestanddelen. Dit betekent dat er dus wel signaalstoffen worden aangemaakt die witte bloedcellen kunnen aantrekken, maar er is uit eerder onderzoek van onze groep gebleken dat er maar zeer weinig witte bloedcellen, en in

het bijzonder neutrofielen granulocyten, in de hersenvloeistof van patiënten met cryptococcose aanwezig zijn.

Al meer dan 50 jaar is bekend dat het polysaccharide kapsel van de cryptococ, en in het bijzonder de belangrijkste bouwsteen daarvan glucuronoxylomannan (GXM), in staat is om het migreren van witte bloedcellen te remmen. In **hoofdstuk 7** hebben we in patiënten met cryptococcose gekeken naar het verband tussen de aanwezigheid van GXM en het ontbreken van witte bloedcellen. Er blijkt dat wanneer de concentraties GXM in bloed ten opzichte van hersenvloeistof - dus de GXM gradiënt over de bloed-hersen-barrière - wordt vergeleken met het absolute cel aantal in de hersenvloeistof, een sterke GXM gradiënt gepaard gaat met weinig cellen in de hersenvloeistof.

Aanvankelijk was het doel van verder onderzoek het achterhalen van het moleculaire mechanisme waarmee GXM in staat is om witte bloedcel migratie naar een signaalstof (chemoattractant) te remmen. Om dit te onderzoeken hebben we *C.neoformans* laten groeien in een vloeibare oplossing met voedingsstoffen (medium). Na 5 dagen groei, waarin de gisten zich vele malen hebben vermenigvuldigd, werden alle gisten verwijderd uit het medium. De overgebleven vloeistof die cryptococcen supernatant wordt genoemd werd gebruikt om GXM uit te isoleren. Geheel onverwacht vonden we dat genetisch gemodificeerde cryptococcenstammen die geen GXM produceren evengoed granulocyten migratie konden remmen als GXM-producerende stammen (**hoofdstuk 8**). Vervolgens hebben we alle bekende kapselcomponenten: galactoxylomannan (GalXM), en de mannoproteinen 1, 2 en 4 (MP1-4) getest op hun capaciteit te interfereren met granulocyten migratie. Uiteindelijk bleek MP-4 de meest krachtige remmer te zijn. We vonden verder dat MP-4 zelf ook in staat is om granulocyten aan te trekken. Deze ogenschijnlijke paradox (het feit dat een zelfde stofje zowel in staat is om granulocyten af te stoten als aan te trekken) wordt verklaard door een fenomeen dat we kruis-desensitisatie (cross-desentization) van receptoren noemen. Dit betekent dat als een witte bloedcel geconfronteerd wordt door een eerste aantrekkende (chemoattractieve) stof, de cel zodanig geactiveerd raakt, dat hij niet meer kan reageren op een tweede chemoattractief signaal. Wanneer we een granulocyt confronteren met een chemoattractieve stof, reageert deze in een reageerbuisje door calcium te mobiliseren. Door dit calcium als het ware van te voren te kleuren met een kleurstof kunnen we dit kleursignaal meten. De kruis-desensitisatie hypothese werd bevestigd door experimenten waarbij we granulocyten incuberen met MP-4 en vervolgens confronteren met een tweede chemoattractieve stof (zoals IL-8, fMLP, en C5a). Bij de tweede confrontatie reageerden de granulocyten niet meer met de gebruikelijke calcium mobilisatie.

Witte bloedcellen vervullen in het lichaam een belangrijke afweerfunctie tegen binnendringende micro-organismen. Echter, in bepaalde gevallen kunnen witte

bloedcellen ook ernstige schade aan het lichaam toebrengen, zoals bij ziektebeelden als bacteriële hersenvliesontsteking, neurotrauma of cerebrovasculaire aandoeningen. Daarom wordt nu aangenomen dat in deze gevallen aanvullende ontstekingsremmende medicijnen neurologisch herstel kunnen bevorderen. Omdat bestanddelen van het cryptococcen kapsel in staat zijn om witte bloedcel migratie te remmen, is onderzoek naar de bruikbaarheid van deze componenten als therapeuticum een belangrijke stap verder. Er blijven echter nog een heleboel vragen te beantwoorden over het mechanisme van (bij)werking. Dit zal ook het onderwerp moeten zijn van toekomstig onderzoek.

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Curriculum vitae

De auteur van dit proefschrift werd op 7 juli 1969 geboren te Breda. Na het behalen van haar VWO diploma aan het College Blaucapel te Utrecht in 1988, ging zij een jaar op reis in Europa. Daarna werkte zij een jaar als verpleeghulp in een bejaarde/verzorgingstehuis in Utrecht, daarnaast deed ze een colloquium natuurkunde aan de Universiteit van Utrecht. In 1990 startte zij met de studie geneeskunde aan de Universiteit van Utrecht. In 1993 werd de studie 8 maanden onderbroken voor een wetenschappelijke stage bij de afdeling Immunologie, vakgroep Infectieziekten en Immunologie faculteit Diergeneeskunde Universiteit Utrecht (hoofd Prof. dr. W. Van Eden) onder begeleiding van Dr. M. Wauben en Dr. I. Joosten en bij het instituut voor Medische Microbiologie en Immunologie (Prof. dr. S. Buus) Kopenhagen, Denemarken. De wachttijd voor de co-schappen van oktober 1994 tot februari 1995 werd gebruikt voor een reis naar India, Nepal en Thailand. In mei 1996 vertrok zij voor het co-assistentschap Gynaecologie en Obstetrie naar Zimbabwe (begeleider: Drs. D.A.A. Verkuyl, United Bulawayo Hospitals, Bulawayo). In maart 1997 werd het artsexamen gehaald, en in mei 1997 begon zij als arts-onderzoeker bij het Eijkman-Winkler Instituut (hoofd: Prof. dr. J. Verhoef) onder begeleiding van Prof. dr. I. M. Hoepelman. In mei 1999 startte zij haar opleiding tot internist in het ziekenhuis Eemland te Amersfoort (opleider: Dr. A. van de Wiel). Vanaf september 2001 werkte zij opnieuw binnen het EWI aan het onderzoek dat wordt beschreven in dit proefschrift, vanaf 1 april 2002 gecombineerd met een tijdelijke functie bij de Stichting Artsen Laboratorium Utrecht /Trombosedienst. Vanaf 1 januari 2003 zal zij haar opleiding tot internist vervolgen binnen het Universitair Medisch Centrum Utrecht (opleider: Prof. dr. D.W. Erkelens).