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Binding and Internalization of Glucuronoxylomannan, the Major Capsular Polysaccharide of *Cryptococcus neoformans*, by Murine Peritoneal Macrophages

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ABSTRACT

Cryptococcus neoformans (C. neoformans) infection is of particular importance in modern medicine. *C. neoformans* is an encapsulated fungus which largely causes opportunistic infections, especially in AIDS or other immunocompromised patients. Due to a strong tropism for the central neural system, *C. neoformans* is the most common cause of fungal meningoencephalitis. Prior to the implementation of HAART (highly active antiretroviral therapy), up to 5-10% of US patients with AIDS developed cryptococcosis. In humans, the encapsulated yeast *C. neoformans* is transmitted by inhalation into the alveolar spaces and causes severe life-threatening pneumonia, meningitis, as well as disease in other organ diseases (cryptococcosis). Furthermore, cryptococcosis is a worldwide disease.

The polysaccharide capsule of *C. neoformans*, the major virulence factor, is composed chiefly of glucuronoxylomannan (GXM) and two minor constituents, galactoxylomannan (GalXM) and mannoprotein (MP). The capsule's interactions with the systems of innate and adaptive immunity modulate the host immune reaction against *C. neoformans* infection.

With the emerging importance of protective immune responses mediated by innate and adaptive immunity, the regulation and molecular mechanisms during *C. neoformans* infection or pathogenesis have begun to unfold.

The two main foci of attention in this study of *C. neoformans* are as follows:

(1) Host immune reactions against *C. neoformans* infection (Chapter 1)

(2) Binding and internalization of glucuronoxylomannan (GXM), the major capsular polysaccharide of *C. neoformans,* by murine peritoneal macrophages (Chapter 2)

Chapter 1 provides an overview of the nature of GXM or *C. neoformans* and recent advances regarding the interaction of the host immune system with GXM or *C. neoformans* that include the Th1-dominated response and complement- and antibody-mediated rapid anti-GXM capsular host defense reactions. Chapter 2 describes the central research aim, which is to examine selected cellular mechanisms for binding and uptake of GXM by murine peritoneal macrophages. I hope that the information from Chapter 1 and Chapter 2 may contribute to an understanding of how to facilitate the host response against *C. neoformans* infection and yield new insights into future immunomodulatory strategies for the treatment of *C. neoformans* infection.

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Chapter 1: A review: *C. neoformans* and host immune responses

Introduction

Despite the presence of approximately 100,000 known species of fungi that inhabit planet Earth, serious disease in humans produced by fungal infections is relatively rare (56). The yeast *Cryptococcus neoformans* is an encapsulated fungus that largely causes opportunistc infections in the central nervous system in immunocomprised individuals (26). During the last 20 years, cryptococcosis ranked fourth world-wide in infectious diseases that lead to death in AIDS patients without treatment by antifungal drugs (77). Cryptococcosis occurs most commonly in AIDS patients, who account for approximately 80-90% of all cryptococcal infections. Prior to the use of HAART, cryptococcosis occured in 5-10% of AIDS patients in the USA, but the incidence of cryptococcosis in AIDS patients in developing countries is much higher (46,83).

The host immune response to *C. neoformans* is diverse, involving innate and adaptive immunity. Chapter 1 reviews the role of innate immune cells, especially phagocytes and complements, against *C. neoformans.* Macrophages are the predominant cells in the process of phagocytosis and the killing of *C. neoformans* (68).

This study also reviews cell-mediated immunity in *C. neoformans* in the belief that the host defense against *C. neoformans* depends primarily on the cell response (56, 77), especially on the protective formation of Th1 polarization and delayed-type hypersensitivity (DTH) (38, 77). Studies indicate that although $CD4^+$ T cell deficiency is critically associated with the development of HIV-related cryptococcosis, an additional factor is involved in B cell dysfunction and/or repertoire defects, or aberrant and unstable expression of immunoglobulin genes of B lymphocytes in HIV-infected patients,

suggesting that the generation of antibodies against antigens of *C. neoformans* is also an important contributor to host immunity (26).

Antibodies enhance opsonization, activating the complement system and leading to increased phagocytosis. These effects facilitate the killing of *C. neoformans* cells and promote the clearance of capsular polysaccharide antigens from the serum. These results demonstrate that the host response to *C. neoformans* is the outcome of interplay among innate immunity and adaptive immunity and fungal virulence factors.

The capsular polysaccharide of *C. neoformans* is composed primarily of the polysaccharide glucuroxylomannan (GXM); the molecular weight ranges from 1.7 to 7.0 X 10^6 daltons from antigenically different strains (67). GXM is a major factor in *C. neoformans* infection (46, 83).

Chapter 1 examines host-induced anti-GXM and/or *C. neoformans* immune responses and GXM and/or *C. neoformans*-mediated immunosuppression. Chapter 1 also reviews how a host helps protect from GXM or *C. neoformans*. These observations suggest an opportunity for the control of *C. neoformans* infection in humans.

History and disease

Cryptococcus was initially identified and isolated from peach juice in 1894. This amount of fermented peach juice introduced to the world and identified the capsulated yeast-like fungus that we now term *C. neoformans* (83, 84). Brusse and Buschke reported that a young woman suffered a chronic ulcer of the skin over her tibia related to *C. neoformans*-mediated infection, which was the first known case of human cryptococcosis (83). It is now evident that *C. neoformans* exists mainly in a saprophytic niche. It has also

been isolated from pigeon droppings and the tree *Eucalyptus camaldulensis* (9, 46). Before 1955, it was a rare human pathogen; just over 300 cases of cryptococcosis were reported in medical journals. For more than 20 years, cryptococcal *C. neoformans* has been recognized as a common worldwide opportunistic pathogen in immunocompromised patients, especially in AIDS, organ transplantation and cancer and, occasionally, in immunocompetent hosts (46, 83).

Three distinct species

C. neoformans strains have been grouped into three varieties that include five serotypes based on capsule structure. It has been proposed that the serotype A strains be classified into the *C. neoformans* var. *grubii;* the serotype D is in *the C. neoformans* var. *neoformans;* the *C. neoformans* var. *gattii* is with the serotypes B and C. However, serotype AD strains possibly occur as incomplete genetic crosses between *neoformans* and *grubii*. The first and second strains are found mainly in temperate climates and are commonly isolated from pigeon feces. The third is found mainly in tropical and subtropical weather conditions and has been isolated from the tree *Eucalyptus camaldulensis* as one of the sources for its environmental niche (46, 83). *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* predominantly infect immunocompromised hosts, and *C. neoformans* var. *gattii* infects non-immunocompromised hosts (10).

Seroptypes based on antigen or molecular structure difference

C. neoformans is a pathogenic yeast which is surrounded by a phagocytosis-inhibiting capsular polysaccharide. The precise structures of GXM were identified by the use of nuclear magnetic resonance techniques, indicating that the capsule structure consists of three polysaccharide components: an abundant glucuronoxylomanan (GXM) making up about 90% of the capsule mass, a minor galactoxylomannan (GalXM) and another minor component, namely mannoprotein (MP). These compounds have some distinct, even somewhat opposite, effects on immune responses to *C. neoformans* (11,17). GXM polysaccharide is a high molecular weight polymer with a linear α-(1→3)-linked mannose backbone and is decorated with non-reducing D-xylosyl and D-glucosyluronic acid groups. In general, two of every three mannose residues are also 6-*O*-acetylated since unbranched mannose prefers being acetylated (11, 44, 50). GXM differs in the degree of xylose addition and acetylation of the mannose that is substituted with glucuronic acid (11). These differences contribute to the classification of four major serotypes, A, B, C, and D (46, 83). Serotype D is the most heavily *O*-acetylated (48, 71). *C.neoformans* with serotypes A and D is found worldwide and causes the cryptococcosis most often observed in AIDS patients (46, 83). In contrast, *C. neoformans* var. *gattii* with serotypes B and C is mostly geographically restricted and is infrequently found in AIDS patients except in some areas of Africa (46, 59, 71). A fifth serotype, AD, is proposed, as some strains display characteristics of both A and D serotypes (41). Serotype A GXM occurs in 99% of AIDS patients with cryptococcosis worldwide. In contrast, in France, around 80% of AIDS patients with *C. neoformans* have serotype A (71).

Several virulence factors

The encapsulated yeast *C. neoformans* has been investigated for more than 50 years and dozens of specific null mutations have been made in examining impacts on the virulence factors of this deadly pathogen (83).

Capsule The polysaccharide capsule, GXM and GalXM, is essential for virulence (46, 83). Several mechanisms have been described by which the capsule and its polysaccharides affect the host response. First the capsule and its polysaccharide can act as a mechanical shield or an antiphagocytosis barrier, interfering with antigen presentation, reducing cytokine production, depleting complement loading, and enhancing HIV replication (46, 83). Current studies show that the capsular polysaccharides GXM and GalXM compromise the host immune response by up-regulated FasL expression to mediate macrophage apoptosis (95). In addition, elevated cryptococcal antigen levels would favor induction of T-suppressor cell response and differentiation towards non-protective Th2-type cytokine response (3). The T-suppressor cell response was associated with a repressed delayed-type hypersensitivity reaction to *C. neoformans* antigen (80). The major capsular component, GXM, also down-regulates the expression of C5a receptor on the surface of human neutrophils (74), inhibiting the C5a-mediated activities of chemoattractance and phagocytosis.

Furthermore, *C. neoformans* is able to change its capsule and structure during infection (phenotype switching), resulting in enhanced virulence (an infectious organism's molecular products for its survival and causing disease in the host) and lethal outcome in mice (29).

Mannitol *C. neoformans* produces a large amount of the hexitol mannitol, which can scavenge toxic products of phagocyte oxidative metabolism, thereby protecting the fungus from oxidative damage by human neutrophils (15).

Several enzymes Several enzymes have been shown to be virulence factors for *C. neoformans,* such as phenol oxidase or laccase (diphenol oxidase). Melanin can be synthesized by a laccase through multiple diphenolic and diamino compounds. The amount of melanin in a melanized cell is sufficient to neutralize a large proportion of cellular oxidants produced by stimulated macrophages or alveolar macrophages and increase resistance to the fungicidal treatment effects of UV irradiation and amphotericin B (14, 46, 52, 60). Passive immunization with a monoclonal antibody to melanin prolonged survival by reducing the fungal burden (14), showing that phenoloxidase-mediated melanin formation is one of several virulence factors.

Phosphlipase may destroy the cell membrane of the host cells (18), and urease is a metalloenzyme that catalyzes the hydrolysis of urea to ammonia and carbamate to induce an increase in pH. Urease induces the production of ammonium hydroxide, which may mediate tissue damage and change the function of the white blood cells (19). In addition, in vivo production of proteinases by *C. neoformans* can also multiply the yeast in the skin and bone and increase its ability to traverse the blood-brain barrier (46).

C. neoformans and innate immunity

The mammalian immune system consists of innate and adaptive immunity. The early innate immune system is the front line of host defense against invading pathogens

such as viruses, bacteria, fungi and parasites. The mechanisms of innate defense include skin and mucosal epithelial surfaces, such as the respiratory and gastrointestinal tracts and so on. The mammalian innate antifungal defense system consists of professional phagocytes (neutrophils, monocytes/macrophages and dendritic cells) and natural killer (NK) cells, γδT cells and non-haematopoetic cells (such as epithelial and endothelial cells) and complements and several humoral factors (87). The specific biological activities of the complement system and antibodies which contribute to host resistance are multifaceted and interdependent, because antibodies contribute to the activation of the complement system by fungi (43) and the complement is essential for antibody-mediated protection (96). Innate immune responses to *C. neoformans* have focused on complement activation and local recruitment of inflammatory cells and secretion of inflammatory cytokines (87).

Complement and phagocytosis

A rapid anti-cryptococcal response can be induced by activation of the host complement system. It has been almost 100 years since the system's initial description as a heat-labile factor "complementing" its killing activity for microbial pathogens (46, 96). After complement activation by pathogens, complement proteins generate fragments and protein complexes which mediate acute inflammatory reactions, clearance of foreign cells and molecules, and killing of pathogenic microorganisms (47). When a pathogen breaches the skin or epithelial cells of the host barriers, it faces the first-line of the innate defense system, the complement cascade. The complement system consists of over 30 proteins in the blood/plasma or cell-surface. Complement activation occurs on a target

surface where the proteins induce target opsonization and clearance, or lysis (47, 96). When a product of the complement cascade or an antibody causes extracellular pathogens or other foreign cells to become more susceptible to destruction by phagocytes (monocytes, macrophages, dendtritic cells and neutrophils), the process of alteration is referred to as opsonization.

Opsonization with complement proteins, in particular with C3-fragments, enhances uptake of microbes into phagocytes via complement receptors. Fragments of C3 are well suited to serve as the source of the major opsonin of the complement system since the C3 concentration in plasma is higher than any other component protein (6). There are three different pathways that lead to the formation of C3 convertases for further cleavage of C3 into small C3a (peptide mediator of inflammation) and large C3b (bind to pathogen surface and act as opsonins) that binds to complement receptor CR1. In addition, factor H binds to C3b and facilitates cleavage of C3b by the plasma protease factor I to produce inactive C3b (iC3b) that binds to complement receptor CR3 on macrophage surfaces. Interestingly, PMA can trigger both CR1 (CD35; C3b receptor) and CR3 (Mac-1, CD11b/CD18; iC3b receptor) activation for phagocytosis (100, 101).

The first pathway, the classical pathway, is activated by the binding of the C1q component to the antibody that is complexed with the antigen on the pathogen. The second pathway, the mannose-binding lectin (MBL) pathway, is triggered by the binding of the serum protein MBL, which is increased during the acute-phase response, to mannose-containing carbohydrates on microbes. Finally, the alternative pathway begins with the binding of the complement protein C3b to *C. neoformans*, but has no requirement of the presence of a specific antibody. The levels of *C. neoformans*-triggered

complement activation are determined by the presence of its capsule and the presence of anti-fungal antibodies in the host serum at the time of infection (45). Because naïve host serum contains insufficient naturally occurring capsule-binding antibodies to activate the classical pathways, the alternative pathway becomes a primary method for activation of complement-induced phagocytosis of encapsulated *C. neoformans* and leads to the deposition of 10^7-10^8 C3 fragments on the surface by each yeast cell (50, 51). Overall, encapsulated *C. neoformans* is a very powerful activator of the complement cascade, with complement activation occurring mainly via the alternative pathway. The purified capsular polysaccharide, glucuronoxylomannan (GXM), also stimulates C3 production by peritoneal cells (9). Complement, antibodies and collectins not only serve as the first line of innate immune response against fungi, but also regulate cytokine secretion and co-stimulatory molecule expression by phagocytes for mediating adaptive immunity (96).

C. neoformans and adaptive immunity

Antigen-presenting cells (APCs)

Protective adaptive immunity to *C. neoformans* is dependent on antigen-presenting cells that mediate Th1-type activation. Antigen-presenting cells (APCs), such as monocytes/macrophages, play major roles as sentinels for initiation of innate immunity and induction of adaptive immune responses (4, 66). Dendritic cells (DCs), regarded as the most professional of the APCs, are able to phagocytose *C. neoformans in vitro* after opsonization by either complement or antibody (42). DCs can also phagocytose *C. neoformans in vivo,* following pulmonary inoculation that leads to DC maturation and antigen presentation, to *C. neoformans*-specific T cells (99). Phagocytosis is an active

process in which the bound pathogen is first surrounded by the phagocyte membrane and then internalized in a membrane-bounded vesicle termed endocytic vacuole or phagosome. The phagosome then becomes acidified, killing most pathogens. Once the phagosome reaches one or more lysosome(s) by fusion to form a phagolysosome, it starts degrading phagocytosed pathogen and antigen-loading to MHC-II for antigen presentation to T cells. In examining the intracellular location of *C. neoformans* yeast cells after phagocytosis by human monocyte-derived DCc, the Levitz lab observed the killing of *C. neoformans* by CD11c positive bone marrow cells and human monocyte-derived DCs (induced by GM-CSF and IL-4 cultivation), finding that *C. neoformans* enters the phagocytosed pathway and is killed by lysosomal components (98). Importantly, endosomes and lysosomes function as antigen storage and present the antigen to T cells. Understanding the interaction of APC and its lysosomal components with *C. neoformans* or GXM should be important in revealing MHC-restricted antigen presentation to naïve T cells. Additionally, these scientists have noted the important role of APCs in potentiating antigen presentation to T cells through complement-sufficient plasma and human anti-GXM antibody-mediated phagocytosis (98).

T lymphocyte and Th1 response resistance to infection

The critical role of specific T cells in anti-*C. neoformans* adaptive immunity can be demonstrated through depletion of $CD4^+$ and $CD8^+$ T lymphocyte subsets in pulmonary clearance experiments (39, 72, 78), as well as with congenitally lymphocyte deficient SCID and athymic mutant strains (40). APCs activate naïve T cells via antigen presentation and cytokine production. Qureshi and his co-workers used a moderately

virulent clinical isolate of *C. neoformans* to infect two genetically distinct strains of mice (C57BL/6J and SJL/J) via the mouse respiratory tract. They found that SJL/J mice are highly resistant to pulmonary and extra-pulmonary *C. neoformans* infection compared to the C57BL/6J mice. Further study found that the resistant phenotype of SJL/J mice is associated with macrophages (or APCs) that mediate Th1-cell-polarized adaptive immune responses through cytokine IFN-γ and IL-12/IL-23p40 and chemokines MIP-1α, MIP-1β and MIP-2 and so on. Conversely, C57BL/6J mice exhibited a Th2 response, as shown by eosinophilia and IL-4 production. Together, these different Th1- and Th2-cell-polarized adaptive immune responses have demonstrated that Th1-cell immune generation is associated with the anti-cryptococcal burden in SJL/J mice. In contrast, the Th2-cell-response leads to a progressive increase of the *C. neoformans* burden in C57BL/6J mice (35). In addition, J.W. Murphy's lab indicated that cell-mediated immunity to *C. neoformans* can be detected by DTH (delayed type hypersensitivity) elicited by an antigen in skin and by Th1-cells in CBA/J mice (27). These results clearly demonstrated that the Th1 response mediated a resistance to infection by *C. neoformans*.

B cells and antibodies against C. neoformans

One of the major functions of the immune system is the production of antibodies by B lymphocytes. Serological studies indicate that *C. neoformans* infections are very common in immunocompetent individuals, including children, but disease is rare, suggesting that antibody-mediated immunity (AMI) against *C. neoformans* may play an important protective role (28, 30). Antibodies can enhance the opsonization and activate the complement system, thereby increasing the killing of *C. neoformans*. In addition,

antibodies promote the clearance of the polysaccharide antigen from the serum. Capsular polysaccharides can elicit either protective or non-protective antibodies. The Kozel lab examined the binding of mAbs to the capsule by differential interference contrast (DIC) microscopy (61, 62). Two major patterns were observed. One is termed "rim": the capsule appears transparent with a highly refractive outer ring. The other major pattern is termed "puffy": the capsule appears opaque and lacks a highly refractive outer "rim." These patterns correlate with antibody biological activity in regards to protection and their effects on complement activation (61, 62). Protective monoclonal IgMs bind with a rim pattern and inhibit C3 deposition through the alternative pathways, whereas non-protective IgMs, producing the puffy pattern, have no effect on alternative pathway C3 binding (61). When antibodies bind to the GXM polysaccharide capsule, they inhibit release of soluble polysaccharide (65).

As described above, although the role of $CD4^+$ and $CD8^+$ T cells in protection against *C. neoformans* is well established, the role of B cells in defense against fungal pathogens has been difficult to unequivocally establish since investigation has not indicated any differences between the outcomes of fungal infections in B cell-deficient and in normal mice (1). However, study indicates that B cells can have a protective effect in systemic cryptococcal infection that may be directly or indirectly involved in stimulating the proliferation of $CD4^+$ T cells and sustaining their response to cryptococcal infection (1). Additionally, B cell immunoglobulin dysfunction is associated with HIV-related cryptococcosis. In these patients, the genetic locus (in chromosome 14) of variable heavy-chain 3 (V_H3) has defects, leading to loss of the function to code and translate an effective antibody to GXM (5, 13).

In addition, the murine derived anti-GXM monoclonal IgG1 antibody, MAb18B7, was examined in a Phase I evolution in patients with disseminated cryptococcosis (53), indicating that antibodies to GXM may mediate a protective immunity. Altogether, based on the studies described above, an antibody-mediated protective immune response involves promoting phagocytosis, the killing of fungal cells, and $CD4^+$ T cells required for antibody-mediated protection (13, 98).

Discussion

This review has mentioned that the main virulence factor of *C. neoformans* is the polysaccharide capsule, which consists primarily of GXM. Variations of GXM structure, such as mannose, xylose, glucuronic acid and *O*-acetyl groups, result in antigenic differences, permitting classification of *C. neoformans* strains into five serotypes, termed A, B, C, D, and AD (83).

The capsular polysaccharide or GXM of *C. neoformans* has been demonstrated to have multiple immunosuppressive effects, including (i) phagocytosis inhibition (48), (ii) dysregulation in cytokine production (93), (iii) complement depletion (63), (iv) induction of suppressive T cells to secrete immunosuppressive factors (8, 9), (v) the phenomenon of antibody unreactiveness and so on (79).

Although this review has addressed the major capsule structure of GXM, which can decrease the function of the host immune system, the minor mannoprotein (MP) of cryptococcal capsular polysaccharide has been identified as an immunodominant antigen because MP stimulated T-cell responses to *C. neoformans* in patients recovered from cryptococcosis and in experimental mice (57). DCs generate from either myeloid or

lymphoid bone marrow progenitors. DCs home in on sites of potential antigen entry where they can avidly internalize, process and present MP in the context of MHC II to $CD4^+$ T cells (64). It is important to note that when DCs are stimulated with *C. neoformans* MP plus unmethylated CpG motifs (synthetic cytosine phosphate guanine (CpG)-containing oligodeoxynucleotides, ODN), this treatment promotes production of TNF-α, IL-1 α, IL-6 and IL-12p70 cytokines and MIP-1α, KC , MCP-1 and IP-10 chemokines and it enhances MP-specific MHC-II restricted $CD4^+$ T-cell responses (20). As described above, DCs mediate $CD4^+$ T cell responses and the secretion patterns of cytokines and chemokines that represent an important function of adaptive immunity to a *C. neoformans* infection.

Monocytes are recruited and differentiated into macrophages at the sites of inflammation. Inflammatory macrophages differ from resident tissue macrophages in the surface expression of different receptors (33). Following this point of view, we are interested in knowing whether inflammatory macrophages will be induced by more GXM taken *in vitro,* although the receptor(s) for GXM binding and internalization are unknown (see Chapter 2). A recent report indicates that GXM uptake by macrophages is mediated by multiple receptors, including pattern recognition receptors such as Toll-like receptors TLR2, TLR4 and other receptors such as CD14, CD18 and FcγRII (73).

Historically, mechanisms of antibody action include toxin and viral neutralization, complement activation, opsonization and antibody-dependent cytotoxicity (through phagocytes and NK cells). It has also been suggested that an interplay between antibody-mediated immunity (AMI) and cell-mediated immunity is required (2, 13).

In general, capsule-specific monoclonal antibodies can promote phagocytosis for *C. neoformans* cells by the classical mechanisms through Fc receptors. Notably, $F(ab)_2$ fragments can promote phagocytosis of *C. neofomans* cells in the absence of a complement, suggesting that an immunoglobulin-mediated change in capsule structure promotes a direct interaction between the capsular polysaccharide and the complement receptor (82,92). A previous study found that human serum from 40 normal adult volunteers contained anti-GXM antibodies. Their frequency of occurrence was 28% for IgG (mostly the IgG2 isotype), 98% for IgM and 3% for IgA. In addition, in 98% of normal subjects, the anti-GXM antibodies have kappa light chains. However, a significant decrease in IgM antibodies and anti-GXM antibodies with kappa light chains was observed in HIV-positive individuals, indicating that HIV may inhibit antibody production against GXM (37).

Additionally, it has been reported that human antibodies to the GXM capsular polysaccharides of *C. neoformans* are also associated with use of the heavy-chain V region V_H3 gene located in human genome chromosome 14. In HIV-infected patients, there is dysregulated V_H3 gene expression that may increase susceptibility to the GXM capsular polysaccharide of *C. neoformans* and increase the incidence of cryptococcosis (28, 85). These results clearly suggest that generation of protective antibodies against fungal antigens is one of the hallmarks of interaction between the host and *C. neoformans*.

GXM capsular polysaccharides are heterogeneous antigens that can induce both protective and non-protective antibodies, suggesting that it is important to determine and use protective antibodies. In 2005, a murine-derived anticryptococcal monoclonal antibody 18B7 was applied to treat patients with cryptococcal meningitis for phase I

evaluation (53). Peptide display technology is also available to identify peptide mimics of GXM epitopes for finding new protective antibodies, including an immunogenic GXM-protein conjugated vaccine (13).

Taken together, in this Chapter we have described how available antibodies have played important roles in host immunoprotection against pathogenic *C. neoformans* or its GXM capsular polysaccharide *in vitro* and *in vivo*, suggesting that anti-GXM antibodies can enhance the killing of *C. neoformans,* and prolong survival in animal models. A better understanding of antibody function and response may provide new strategies for future diagnosis, prophylaxis and treatment for *C. neoformans* infection.

The interaction between host and *C. neoformans* is a complex cascade of events, which involves not only the host immune response (humoral and cellular immunity or innate and adaptive immunity) against *C. neoformans,* but also *C. neoformans*-mediated immune evasion mechanisms. This review mainly discusses the mechanisms of the host in resistance to infection through innate and adaptive immunity. Due to space limitations, this review cannot describe the interaction between NK and NKT cells or endothelial cells (which can enhance phagocyte-mediated anti-cryptococcal activity) and *C. neoformans* cells, but we can find their role in a number of different references and books, such as reference (97).

Overall, this review provides an overview of the history and molecular structure in order to understand innate and adaptive immunity related to *C. neoformans* infections. This chapter hopes to have provided key sources of information involving interactions of host immune reactions with *C. neoformans,* including its major polysaccharide component, GXM. Obviously, a better understanding of the interaction between host and

C. neoformans may lead to new strategies for basic research and clinical requirements for host against *C. neoformans*.

Chapter 2: Binding and internalization of glucuronoxylomannan, the major capsular polysaccharide of Cryptococcus neoformans, by murine peritoneal macrophages

Summary

Glucuronoxylomannan (GXM), the major component of the capsular polysaccharide of *C. neoformans*, is essential to the virulence of the yeast. Previous studies found that the interaction between GXM and phagocytic cells has biological consequences that may contribute to either resistance to cryptococcul disease or the pathogenesis of cryptococcosis. We found that GXM binds to and is taken up by murine peritoneal macrophages. Examination of the sites of GXM accumulation by immunofluorescence microscopy showed that the pattern was discontinuous and punctuated both on the surface of macrophages and at intercellular depots. Although resident macrophages showed appreciable accumulation of GXM, uptake was greatest with thioglycollate–elicited macrophages. A modest stimulation of GXM binding followed treatment of resident macrophages with phorbol 12-myristate 13-acetate, but treatment with lipopolysaccharide or gamma interferon alone or in combination had no effect. Accumulation of GXM was critically dependent on cytoskeleton function; a near complete blockade of accumulation followed treatment with inhibitors of actin. GXM

accumulation by elicited macrophages was blocked by treatment with inhibitors of tyrosine kinase, protein kinase C, and phospholipase C, but not by inhibitors of phosphatidylinositol 3-kinase, suggesting a critical role for one or more signaling pathways in the macrophage response to GXM. Taken together, the results demonstrate that it is possible to experimentally enhance or suppress binding of GXM to macrophages, raising the possibility for regulation of the interaction between this essential virulence factor and the binding sites on cells that are central to host resistance.

Introduction

C. neoformans is an opportunistic fungal pathogen that may cause a life-threatening meningoencephalitis in immunocompromised patients, especially individuals with AIDS. The *C. neoformans* cell is surrounded by a polysaccharide capsule that is its major virulence factor (12). The primary constituent of the capsular polysaccharide of *C. neoformans* is glucuronoxylomannan (GXM), a high-molecular weight polysaccharide that can be isolated from the supernatant fluid of live *C. neoformans* cultures and may be found in high concentrations in serum and cerebrospinal fluid from patients with cryptococcosis. GXM is composed of mannose, xylose, glucuronic acid and *O*-acetyl groups (7, 17)**.**

There are several direct and indirect lines of evidence for the binding and/or uptake of GXM by phagocytes. First, large amounts of GXM are shed *in vivo*, and immunohistochemistry studies have found that GXM accumulates and is stored in tissue macrophages (31, 34, 76). Second, soluble GXM blocks binding of CD18 antibodies to human neutrophil CD18 (23). Third, Shoham et al. found that GXM bound to Chinese

hamster ovary (CHO) cells that are transfected with Toll-like receptors 2 and 4 and/or CD14. Fourth, GXM interactions on the yeast surface with phagocyte receptors such as CR3 (CD11b/CD18) and CR4 (CD11c/CD18), CD14, TLR2 and TLR4, individually or in combination, may be responsible for this process (82, 92). Finally, previous study directly demonstrates binding and uptake of GXM by human monocytes and neutrophils (75).

Macrophages were first described by Elie Metchnnikoff in the 1880's. He received the Nobel Prize in Medicine and Physiology in 1908 for his studies of phagocytosis (81). Macrophages are thought to be assigned a function in host defense and remain a hot topic in immunology, cell biology and experimental medicine today. Macrophages mediate phagocytosis and generate proinflammatory mediators such as various cytokines, chemokines, and reactive oxygen species (66).

Macrophages occupy a pivotal position in the host immune system because they play an important role in host defense as an effector cell to phagocytose, digest, and kill bacteria, fungi, and virus pathogens. Macrophages are also critical regulatory cells that mediate antigen presentation, cytokine secretion and other secretory activities to induce positive and negative inflammatory responses and adaptive immunity.

In 1995, 1998 and 2001, the tissue location of GXM and clearance *in vivo* were reported (31, 34, 54), and these immune phenomena seem to suggest that macrophages may play a critical role in directly uptaking and digesting GXM *in vivo*. Conversely, the binding and/or uptake of GXM by macrophages may have additional biological consequences that contribute to the pathogenesis of cryptococcosis. The potential consequences of GXM-phagocyte interaction include cryptococcal polysaccharides, or

GXM, mediated inhibition of neutrophil influx into sites of inflammation (22), inducing shedding of L-selection from neutrophils (23) and blockading of the CD18 dependent leukocyte-endothelial cell interaction and infiltration into inflammatory sites (23). GXM mediated inhibition also induces alternations in cytokine production by leukocytes (21, 86, 93, 94)**,** blocking of interaction between yeast-bound GXM and phagocyte receptors for GXM (82)**,** and decreased killing of encapsulated and acapsular cryptococci (75).

Despite abundance evidence for GXM-phagocyte interaction, little is known of cellular events that occur on binding or uptake of GXM by phagocytes. The major goals of this study were to further characterize the factors that have an effect on attachment and ingestion of GXM by macrophages. The objectives of our study were to (i) establish the kinetics for binding and uptake of GXM, (ii) identify the cell pattern of GXM binding, (iii) determine the requirements for the cytoskeleton, (iv) assess the requirements for macrophage activation, and (iv) examine the involvement of signal transduction pathways.

MATERIALS AND METHODS

Cryptococcal polysaccharide GXM was isolated from culture supernatant fluid of *C. neoformans,* serotype A strain CN6 that was provided by Dr. R. Cherniak (Georgia State University, Atlanta, GA). Yeast cells were grown in a synthetic medium (17) for 4 days at 30ºC. The GXM was isolated and purified from culture supernatant by differential precipitation with ethanol and cetyltrimethylammonium bromide (CTAB) (16). A stock solution of GXM in phosphate-buffered saline (PBS) at a concentration of 8 mg/ml was made, sterilized by filtration, and kept at 4ºC. Isolated soluble GXM produced a negative

result in LPS testing by the Limulus amebocyte lysate test (QCL-1000; Cambrex Bio Science, Walkersville, MD).

GXM Monoclonal Antibody (MAb) MAb 3C2 was produced from splenocytes obtained from BALB/c mice (Simonsen Laboratories, Gilroy, CA) immunized with serotype A GXM coupled to sheep erythrocytes. The immune spleen cells were fused with Sp2/0-Ag14 mouse myeloma cells (25). MAb 3C2 was isolated from the ascites fluid by differential precipitation with caprylic acid and ammonium sulfate followed by affinity chromatography on a GXM-AH Sepharose column (49) and was further purified by affinity chromatography on protein A. MAb3C2 was coupled to horseradish peroxidase (HRPO) by use of a peroxidase labeling kit (EZ-Link activated peroxidase kit; Pierce, Rockford, IL) according to the direction of the manufacturer. MAb3C2 has been identified as an IgG_1 isotype that is reactive with GXM of *C. neoformans* serotypes A, B, C, and D (90). MAb 3C2 was also coupled to fluorescent dyes Alexa Fluor 555 and Alexa Fluor 488 (Molecular Probes, Eugene, OR) according to the procedures of the manufacturer.

Murine peritoneal macrophages Female Swiss Webster mice were purchased form the Animal Production Program, Frederick Cancer Research & Development Center (Frederick, MD) and utilized at 8 to 10 weeks of age. Before collecting resident peritoneal macrophages, mice were asphyxiated with CO_2, the peritoneal cavity was injected with 5 ml of ice-cold DPBS (Dulbecco's phosphate-buffered saline; Cellgro, Mediatech, Inc., Herndon, VA), and the peritoneal cells were harvested. Elicited macrophages were collected at the third day following injection of 1 ml of autoclaved 10% thioglycollate medium (Sigma-Aldrich, St. Louis, MO). The cells were washed two

times with DPBS and resuspended in Iscove's modified Dulbecco's medium (Cellgro, Mediatech, Inc).

Analysis of GXM binding to macrophages by an enzyme-linked immunosorbent assay (ELISA) To analyze the binding of GXM to macrophages, tissue culture plates (96-well plates; Falcon 35307, Becton Dickinson Labware, Franklin Lakes, NJ) were used for seeding of peritoneal cells (1.25 x 10^5/well) in a culture medium of Iscove's modified Dulbecco's medium containing 10% fetal bovine serum (Cellgro, Mediated, Inc) and 100 μg/ml kanamycin (Sigma-Aldrich) and incubated at 37ºC in a humidified atmosphere with 5% CO_2. After 1 day of incubation, the nonadherent cells were removed by gently washing and incubated for another 24 h. To measure GXM binding, three protocols were used: (i) total GXM bound to macrophages, (ii) GXM bound to the cell exterior, and (iii) GXM entered into the cell interior. In all cases, macrophage monolayers were incubated with various concentrations of GXM for the time indicated for each experiment. To detect total binding of GXM, the cells were washed three times with PBS, fixed in 4% paraformaldehyde for 10 min on ice , and washed three times with PBS. The cells were permeabilized by treatment with 0.1% saponin for 30 min at room temperature and washed an additional four times with PBS. Potential nonspecific binding sites were blocked by incubation of monolayers for 30 min at room temperature with 2.5% bovine serum in PBS (blocking buffer). Binding of GXM was identified by incubation of macrophages for 30 min at room temperature with a working dilution (1μg/ml) of horseradish peroxidase-labeled MAb3C2 (HRPO-MAb3C2) in blocking buffer. Preliminary experiments established that the HRPO-labeled antibody was not limiting at the working dilution. After incubation with HRPO-MAb 3C2, the wells were washed four

times with PBS containing 0.05% Tween 20 and incubated for 30 min at room temperature with 100 μl of 3, 3',5, 5'-tetramethylbezidine peroxidase substrate solution (Kirkegaard & Perry Laboratories; Gaithersburg, MD). The reaction was stopped by addition of 100 ml of 1 M H_3PO_4, and the absorbance at 450 nm was read with an ELISA plate reader (Versamax; Molecular Devices, Sunnyvale, CA). Results are reported as the optical density at 450 nm (OD_{450}) after correction for background. Background levels were characteristically $\leq$ 0.07. Different experiments often utilized different lots of HRPO-labeled antibody. Since the labeling efficiency varied somewhat from one labeling to another, the OD_{450} observed for one experiment often cannot be directly compared to optical densities observed in other experiments. However, when data from replicate experiments were pooled, the data represented experiments done with the same lot of labeled MAb.

Binding of GXM to the exterior of the macrophages was done as described above for determination of total GXM binding with the exception that permeabilization with saponin was omitted. To detect the amount of GXM inside macrophages, macrophage monolayers were treated with GXM as described above. The macrophages were fixed with paraformaldehyde, and nonspecific sites were blocked with blocking buffer for 30 min at room temperature. GXM bound to the macrophage surface was blocked by incubation with unlabeled MAb 3C2 (10 μg/ml) for 30 min at room temperature. Preliminary experiments demonstrated that such treatment reduced binding of HRPO-MAb 3C2 to nonpermeablized GXM-treated macrophages by >95%. The cells were then permeabilized with saponin and incubated for 30 min with blocking buffer, and the

amount of intracellular GXM was determined by using HRPO-MAb 3C2 as described above.

Analysis of GXM binding by confocal microscopy To determine and compare the levels of the GXM binding on the cell surface or localized at the interior of macrophages, peritoneal exudate cells (2.5 x 10^4/ml), prepared as described above, were seeded to each chamber of an eight-well glass chamber slide (Nalge Nunc International Corp., Naperville, IL). After incubation for 24 h, the chambers were washed three times with culture medium to remove nonadherent cells. Next, the medium was replaced, and the monolayers were incubated for an additional 24 h. Macrophage monolayers were treated with GXM (200μl, at 80μg/ml) for 4, 16, 64, and 256 min. Subsequently, the medium was removed and the cells were washed three times with PBS. The macrophages were fixed with paraformaldehyde as described above, and nonspecific binding sites were blocked by treatment for 30 min by blocking buffer. The binding of GXM to the cell surface was stained by incubation for 30 min at room temperature with Alexa Fluor 555-labeled MAb 3C2 (5 μg/ml). Macrophages were then permeabilized by incubation with saponin as described above, and nonspecific binding sites were blocked by treatment with blocking buffer for 30 min at room temperature. GXM located in the interior of the macrophage was stained by incubation with Alexa Fluor 488-MAb 3C2 (5 μg/ml) for 30 min at room temperature. Preliminary experiments demonstrated that preincubation of paraformaldehyde-fixed macrophages with unlabeled MAb 3C2 prevented subsequent binding of Alexa 555-MAb 3C2 to the cells. After the cells were stained, the slides were overlaid with Vectashield (Vector laboratories, Inc., Burlingame, CA) and covered with a coverslip. The binding of distinct fluorescently labeled antibodies was detected with a

Nikon confocal microscope C1 unit that was fitted to a Nikon Eclipse E8000 microscope equipped with different interference contrast optics. Confocal images were obtained with Nikon EZ-C1 software, version 1.70. Merging and cropping of images were done with Simple PCI (Compix, Inc., Cranberry Township, PA).

Effects of activators and inhibitors Ro 31-8220, calphostin C, rottlerin, genistein, 4-amino-5-(4-methylphenyl)-7-(*t*-utyl) pyrazolo(3,4-D)pyrimidine (PP1), 4-amino-5-(4-chlorophenyl)-7-(*t*-utyl) pyrazolo(3,4-D)pyrimidine (PP2), piceatannol, LY294002, wortmannin, dolastatin 15, colchicine, U73122, cytochalasin D, latrunculin A, and vinblastine sulfate were purchased from Biomol (Plymouth Meeting, PA). All of the preceding agents were dissolved in either dimethyl sulfoxide (DMSO) or water and stored at -20ºC. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich and maintained as a stock solution in DMSO. Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5, recombinant gamma interferon (IFN-γ) from mouse and *N*-formyl-Met-Leu-Phe (fMLP) were obtained from Sigma-Aldrich. Each agent was used at the concentration indicated for each experiment in 200 μl of culture medium per well. The final concentration of DMSO for those agents where DMSO was used was ≤0.5%. Control experiments showed that DMSO in concentrations of 2.5% had no effect on the accumulation of GXM by elicited macrophages. Monolayers were prepared as described above for assessment of GXM binding by ELISA and preincubated with inhibitors or activators for the time indicated for each experiment. Unless otherwise indicated, macrophages were preincubated for 30 min with each inhibitor. GXM was then added in a 20-μl volume to achieve a final concentration in the medium of 80 μg/ml, and the incubation was continued for an additional 2 h or an alternative time as required for a

given experiment. The cells were then washed, fixed, and permeabilized as described above, and the amount of total bound GXM was determined by using HRPO-MAb 3C2 as described above.

All experiments that examined the effects of pharmacologic activators or inhibitors were accompanied by control experiments that assessed the effects of the inhibitors on cell viability. Conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetraolium bromide (MTT) (Sigma-Aldrich) by living cells was used as a quantitative measure of cell metabolic activity and viability as described previously (32). Assessment of MTT reduction in the presence of various inhibitors was done in parallel with GXM binding studies under identical conditions, including the presence of GXM. MTT activity was measured after the cells were washed to remove GXM.

Western blot analysis of PI3K activity The abilities of wortmannin and LY294002 to inhibit phospatidylinositol 3-kinase (PI3K) were confirmed by analysis of the effects of the inhibitors on LPS-induced phosphrylation of Akt by PI3K, which is sensitive to wortmannin and LY294002. Elicited macrophages were prepared in 96-well plates as described above. The macrophages were unstimulated or stimulated for 30 min with LPS (10μg/ml) in the presence or absence of wortmannin (400 nm) or LY294002 (10μM). Cell lysates were prepared by use of mammalian protein extraction reagent (Pierce), and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Phosphorylation of Akt at Ser^{473} was assessed by use of a phosphorylated Akt antibody (Catalog no. 9276; Cell Signaling Technology, Inc., Beverly, MA) followed by incubation with alkaline phosphatase-labeled rabbit anti-mouse secondary antibody and alkaline phosphatase substrate

(Promega, Madison, WI). An antibody that bound to both phosphorylated and nonphosphorylated Akt (Catalog no. 9272; Cell Signaling Technology, Inc. Beverly, MA) was used as a control for sample loading.

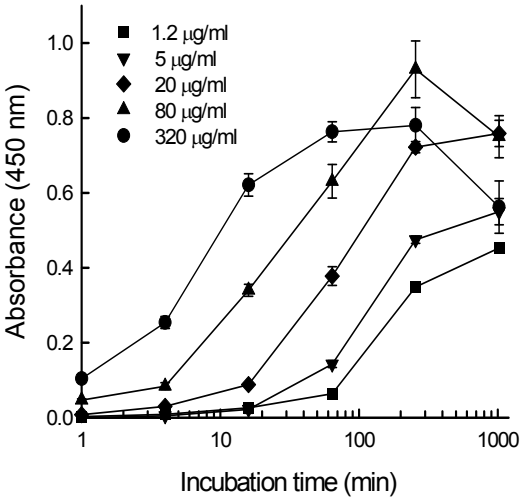
Statistics Comparisons of individual treatment effects with the medium control were made by analysis of variance with a post hoc assessment of individual treatment effects done by the Tukey test. Each experiment was done at least three times with four replicates per experiment. Data were combined and reported as the means ± standard deviations (SDs) when the experimental designs were identical for replicate experiments and the same set of reagents was used. Data from a representative experiment are reported in those instances where replication of the experiment involved slight variation in the experimental design, e.g., incubation times or doses of reagents, or when different lots of reagents were used, e.g., different batches of HRPO-MAb.

Results

Binding and uptake of GXM by elicited macrophages

We first used ELISA to evaluate the binding and uptake of GXM by elicited macrophages. Monolayers of macrophages were incubated for 1, 4, 16, 64, 256 and 1024 min with 200 μl of GXM at concentrations of 1.25, 5, 20, 80, and 320 μg per ml of culture medium. The analysis of dose and time course responses for accumulation of GXM indicated gradual binding of GXM over time for all doses of GXM.

Fig. 1. Time course for accumulation of GXM by elicited peritoneal macrophages. Monolayers of macrophages were incubated with the indicated concentrations of GXM for 1, 4, 16, 64, 256 or 1024 min, and the total amount of bound GXM was determined. Results are reported as the mean ± SDs (error bars) of four replicates for each data point. Results shown are from one of three independent experiments with similar results.

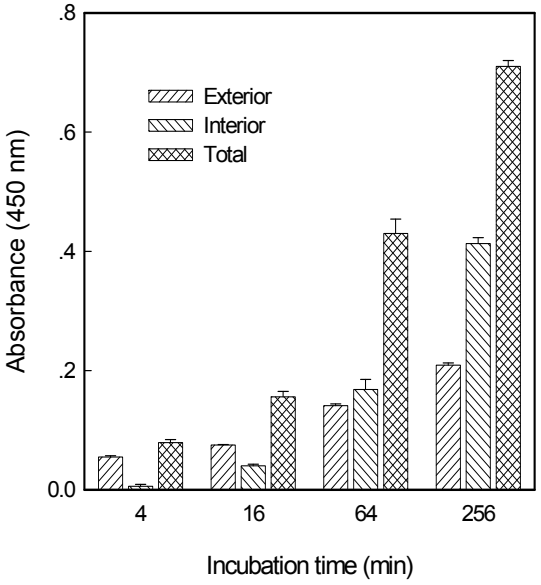


Accumulation was most rapid at the highest concentration of GXM, reaching a maximum uptake followed 16 to 64 min of incubation. The macrophages incubated with GXM at 20 and 80 μg/ml approached maximum levels similar to those observed at the highest concentration, 320 μg per ml, however, at much longer incubation times (256 to 1024 min, respectively) when compared to 320 μg per ml (Fig.1).

The results shown in Fig.1 reflected only the total amount of GXM bound to macrophages but did not indicate whether GXM was located on the exterior or in the interior of the cells. Thus we next evaluated whether GXM was located on the exterior and/or in the interior of the cells. For this purpose, the monolayers of macrophages were incubated with GXM for 4, 16, 24 and 256 min with a dose at 80 μg/ml as described in Fig.1. The results of assays that examined total GXM binding (Fig.2) were similar to the results shown in Fig. 1. Following 4 min incubation, most bound GXM was at the cell exterior. After 64 min of incubation, approximately equal amounts of GXM were found on the cell surface and in the interior. However, after 256 min of incubation, the majority

of the GXM was intracellular, but an appreciable amount of GXM was still bound to the cell surface. This result demonstrates that GXM is found at both the exterior and interior of macrophages.

Fig. 2. Time course for (i) total binding of GXM to elicited peritoneal macrophages, (ii) binding of GXM to the macrophage exterior or (iii) accumulation of GXM in the cell interior. Macrophages were incubated with GXM at 80 μg/ml for the indicated times, and the amount of total, exterior or interior binding was assessed as described in the methods. Results are reported as the mean ± SDs (error bars) of four replicates for each data point. Results shown are from one of four independent experiments with similar results.



Results in Fig. 2 do not provide qualitative information related to the pattern of GXM binding. As a consequence, an experiment was done to assess the sites of GXM binding using an experimental design in which macrophage monolayers were incubated for 4, 16, 64, or 256 min with GXM at 80 μg/ml. The cells were fixed with paraformaldehyde, and the sites of exterior binding were identified by incubation with Alexa Fluor 555-MAb 3C2 (red). We used a high concentration of antibody (5 μg/ml) in an effort to completely block all GXM located on the cell surface. The cells were then permeabilized with saponin, and the sites of interior binding were identified by incubation with Alexa Fluor 488-MAb 3C2 (green). The results (Fig.3) largely paralleled the results shown in Fig.2. GXM was primarily located on the cell surface after 4 min of incubation. With increasing

incubation time, there was an increase in the amount of GXM found in the cell interior such that the majority was intracellular after 256 min. Perhaps the most striking feature of the sites of GXM binding was the punctuate pattern of localization. This discontinuous punctuate pattern was observed for both extracellular and intracellular GXM. In no instance was there a continuous distribution of GXM either on the surface or in the cell interior. Many cells showed sites for accumulation of GXM that were considerably larger than the GXM deposited in the punctuate pattern. Finally, there was considerable cell-to-cell variability in the relative amount of bound GXM; some cells showed considerable amounts of bound GXM, while other cells showed little or no bound GXM, suggesting that the elicited population of macrophages is heterogeneous both developmentally and functionally (88).

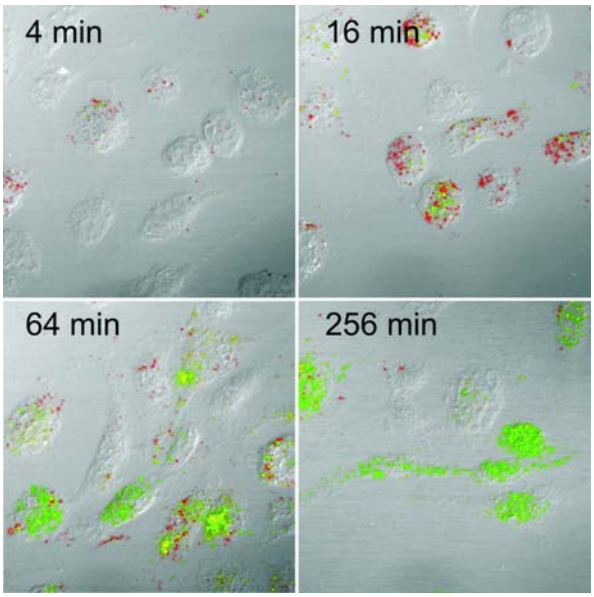


Fig. 3. Cellular sites for GXM bound to the exterior and interior of elicited peritoneal macrophages. Macrophages were incubated with GXM at 80 μg/ml for the indicated times, and GXM bound to the exterior or interior of the cells was determined as described in the Materials and Methods. GXM bound to the exterior of the cells is shown by binding of

Alexa Flour 555-MAb 3C2 (red). GXM bound to the interior of the cells is shown by binding of Alexa Flour 488-MAb 3C2 (green).

Contribution of macrophage activation to GXM accumulation

Results from experiments shown in Figs. 1 to 3 were done with thioglycollate-elicited macrophages. Intraperitoneal injection of thioglycollate broth induces an influx of cells from a pool of preformed monocytes. The biological activities of thioglycollate-elicited macrophages differ from resident peritoneal macrophages in several aspects, including increased metabolic activity and phagocytosis via complement receptors. As a consequence, we examined differences in GXM accumulation between resident macrophages, elicited macrophages, and resident macrophages that were activated by treatment in vitro with various doses of PMA, LPS, IFN-γ in combination with LPS, and fMLP. The results (Fig.4) showed that elicited macrophages accumulated a significantly larger amount of GXM (about two-fold) than did resident macrophages. Stimulation of resident macrophages with PMA produced a significant dose-dependent increase in GXM accumulation. LPS, LPS in combination with IFN-γ, and fMLP had no significant effect on accumulation of GXM by resident macrophages.

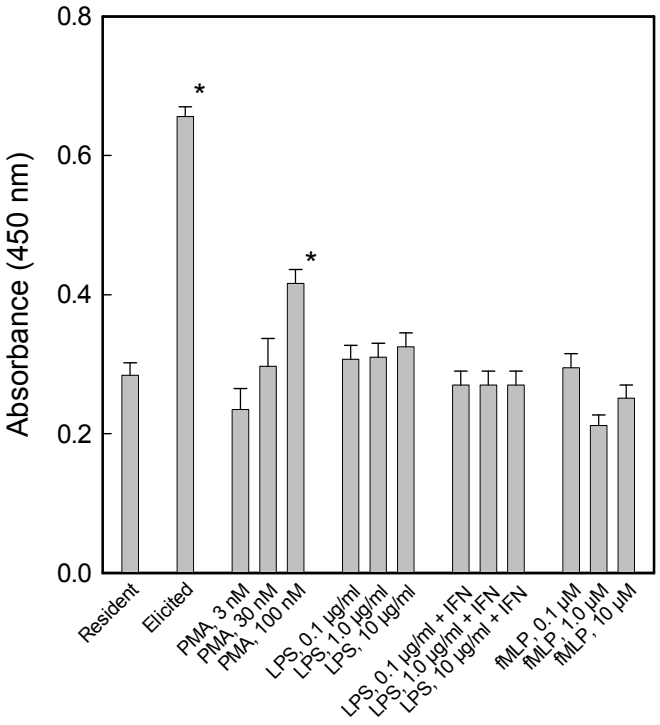


Fig. 4. Effect of macrophage activation on accumulation of GXM by macrophages. Thioglycollate-elicited or resident peritoneal macrophages were collected. Resident macrophages were preincubated for 18 h with LPS, LPS + IFN-γ or fMLP. Pretreatment time with PMA was 45 min (101). Following pretreatment with each potential activator or with medium alone, GXM was added to produce a final concentration of 80 µg/ml and incubated for an additional 2 h at 37ºC. Bound GXM was assessed by ELISA, and the results are reported as the OD_{450}. Data are reported as the mean ± SD (error bars) from three independent experimentents. Values that are significantly different ($P < 0.05$) from the value for untreated resident peritoneal macrophages (*) are indicated.

Requirements for cytoskeleton in accumulation of GXM by elicited macrophages

Inhibitors of actin and tubulin were used to assess the requirements for cytoskeleton in the accumulation of GXM by elicited macrophages. We evaluated the effects of two inhibitors of actin filament function, cytochalasin D and latruculin A, and one inhibitor of microtubule activity, vinblastine sulfate. Inhibitory activity was examined over a broad range of inhibitor concentrations. The results (Fig. 5) show a near complete inhibition of GXM accumulation by both cytochalasin D and latruculin A in a dose-dependent fashion. Substantial inhibition of GXM accumulation was noted with vinblastine; however, the level of inhibition was less than that observed for cytochalasin

D and latruculin A. Evaluation of the effects of each inhibitor on macrophage viability by use of the MTT assay showed no significant (P>0.05) loss of MTT reduction activity by any inhibitor at the concentrations used in the study (data not shown).

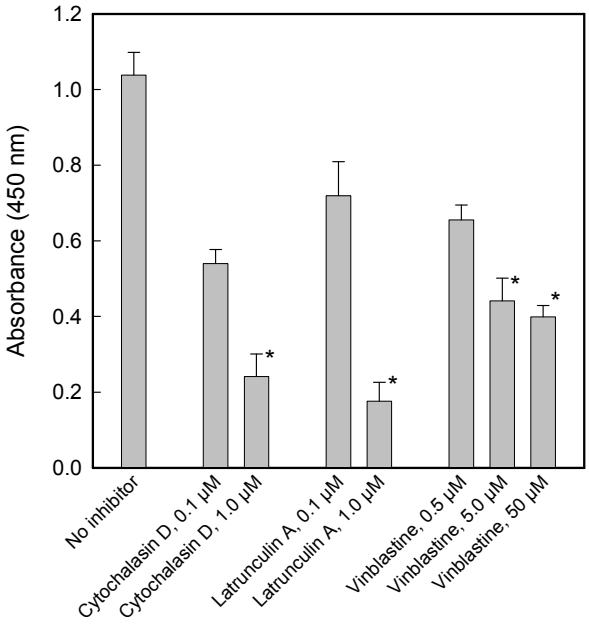


Fig. 5. Effects of inhibitors of cytoskeleton function on accumulation of GXM by elicited macrophages. Elicited macrophages were preincubated with each inhibitor at the indicated concentration or medium alone for 30 min, and GXM was added to produce a final concentration of 80 µg/ml and incubated for 2 h at 37ºC. Bound GXM was assessed by ELISA, and the results are reported as the OD_{450}. Results are the combined data from two independent experiments and are reported as the mean ± SDs (error bars). Values that are significantly different (P < 0.05) from the value for the control with no inhibitor (*) are indicated.

There was only partial inhibition of GXM accumulation by the tubulin inhibitor vinblastin sulfate. As a consequence, we evaluated additional tubulin inhibitors in an effort to determine whether partial inhibition was unique to the general group of tubulin inhibitors. The results (Fig. 6) showed that vinblastin, dolastatin 15, and colchcine all produced a significant (P<0.05) inhibition of GXM accumulation, but the level of inhibition never exceeded 50%. Evaluation of the effect of each inhibitor on cell metabolic activity/viability in the MTT assay showed no significant effect by any inhibitor (data not shown).

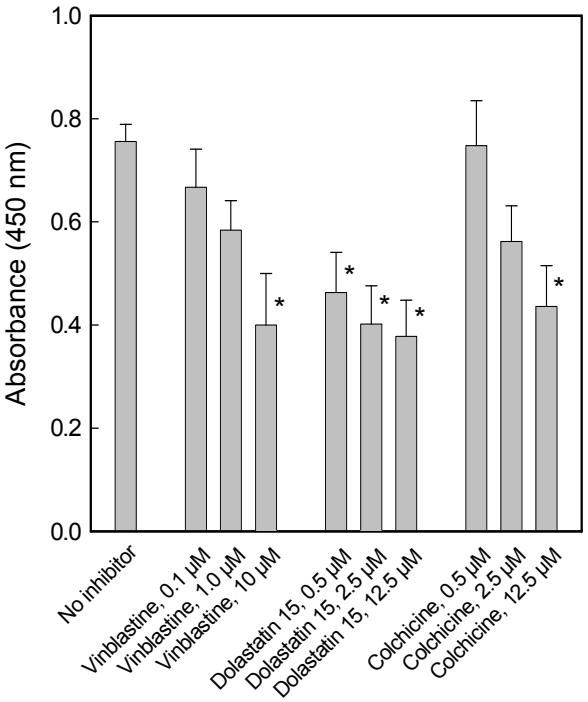


Fig. 6. Effects of inhibitors of tubulin function on accumulation of GXM by elicited macrophages. Macrophages were preincubated with each inhibitor at the indicated concentration or medium alone for 30 min, and GXM was added to produce a final concentration of 80 μg/ml and incubated for 2 h at 37ºC. Bound GXM was assessed by ELISA, and the results are reported as the OD_{450}. Results are the combined data from two independent experiments and are reported as the mean ± SDs (error bars). Values that are significant different ($P < 0.05$) from the control with no inhibitor (*) are indicated.

Effects of pharmacologic inhibitors of signal transduction on accumulation of GXM by elicited macrophages

Internalization of exogenous materials by cells may involve multiple signal transduction pathways. As a consequence, we examined the effects of inhibitors of protein kinase C (PKC), PI3K, phospholipase C, and selected protein tyrosine kinase (PTK) inhibitors on the accumulation of GXM by elicited macrophages. All PKC inhibitors produced near complete inhibition of GXM accumulation in a dose-dependent manner (Fig.7). Evaluation of the effects of each PKC inhibitor on macrophage viability by use of the MTT assay showed no significant loss of MTT reduction activity at any inhibitor concentration that was used ($P<0.05$; data not shown).

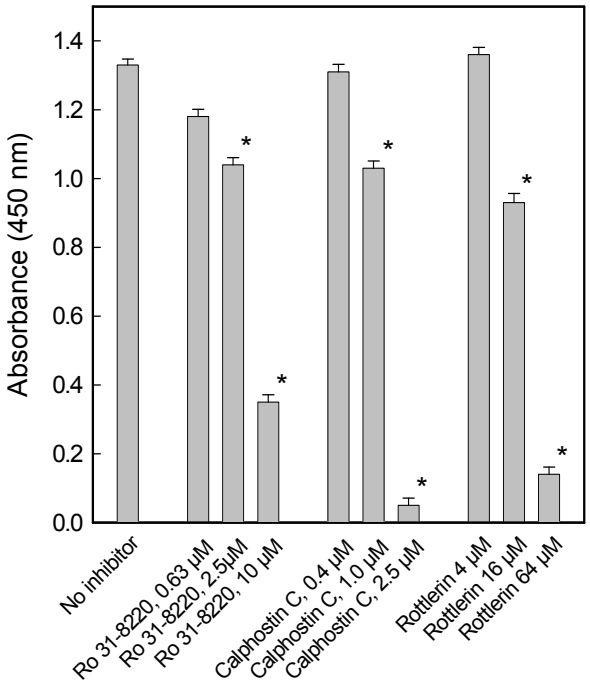


Fig. 7. Effects of protein kinase C inhibitors on accumulation of GXM by elicited macrophages. Macrophages were preincubated for 30 min with each inhibitor at the indicated concentration or medium alone, and GXM was added to produce a final concentration of 80 μg/ml and incubated for 2 h at 37ºC. Bound GXM was assessed by ELISA, and the results are reported as the OD_{450}. Results are the combined data from three independent experiments and are reported as the mean ± SDs (error bars). Values that are significantly different ($P < 0.05$) from the value for the control with no inhibitor (*) are indicated.

The protein tyrosine kinase inhibitors selected for study showed significant ($P<0.05$) inhibition of GXM accumulation (Fig. 8). The Src family inhibitors PP1, PP2, and piceatannol were particularly potent inhibitors, producing a near complete inhibition at the highest inhibitor concentrations. Evaluation of the effects of each inhibitor on macrophage viability by use of the MTT assay showed a significant reduction in activity in the presence of 20 μM PPI (24%) and 40 μM PPI (42%) (data not shown). As a consequence, a portion of the inhibitory activity of PPI may be attributed to a loss of cell activity or viability. No other inhibitors produced a significant inhibition; however, a significant increase in MTT activity was noted for macrophages incubated with GXM in the presence of 100 μM and 400 μM genistein.

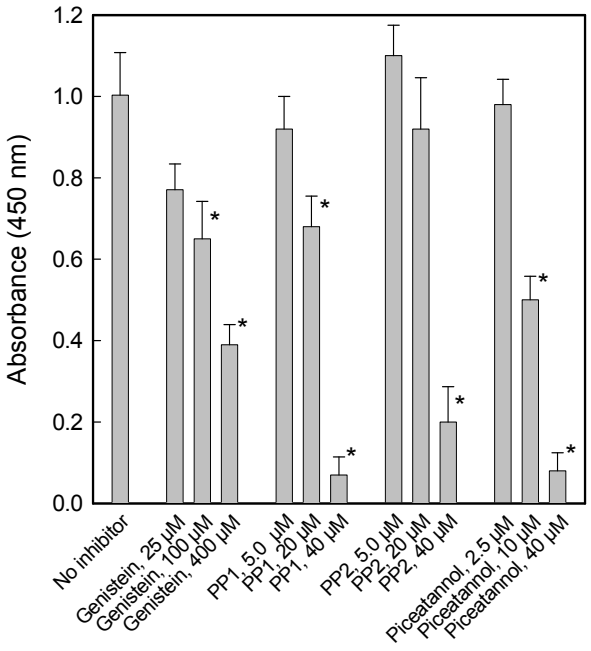


Fig. 8. Effects of tyrosine kinase inhibitors on accumulation of GXM by elicited macrophages. Macrophages were preincubated with each inhibitor at the indicated concentration or medium alone for 30 min, and GXM was added to produce a final concentration of 80 μg/ml and incubated for 2 h at 37ºC. Bound GXM was assessed by ELISA, and the results are reported as the OD_{450}. Results are the combined data from two independent experiments and are reported as the mean ± SDs (error bars). Values that are significantly differently ($P < 0.05$) from the value for the control with no inhibitor (*) are indicated.

Results from three independent experiments found that the PI3K inhibitors LY294002 and wortmannin had little or no effect on the accumulation of GXM by elicited macrophages (data not shown). Given this negative result, we examined the effects of the inhibitors on constitutive and LPS-induced phosphorylation of Akt, which is on a wortmannin- and LY294002-sensitive pathway involving PI3K. The results showed a near complete absence of constitutive or LPS-induced phosphorylation of Akt at Ser ^{473} in the presence of wortmannin (400 nM) or LY294002 (10 μM).

Finally, we examined the effect of the phospholipase C inhibitor U-73122 on the accumulation of GXM by elicited macrophages. The results of three independent experiments showed that U-73122 at concentrations of 1, 3, and 9 μM reduced GXM accumulation by 35%, 48%, and 73%, respectively, relative to uninhibited control macrophages. Examination of the effect of U-73122 on cell viability using the MTT

assay showed no significant difference at any concentration of U-73122 that was used in the experiment.

Discussion

In vivo and *in vitro* studies have provided abundant evidence for the binding of GXM to macrophages. Moreover, the interaction of GXM with macrophages has biological consequences. The goal of our studies was to examine selected cellular mechanisms for binding and uptake of GXM macrophages. We found that elicited macrophages gradually accumulate GXM over time (during 1, 4, 16, 256 or 1024 min) and bind GXM at different concentrations (Fig.1) by ingestion, confirming results reported by Monari et al. who examined the interaction between GXM and human monocyte-derived macrophages (75). The primary new findings of our study are as follows. (i) Cellular sites for GXM binding exhibit a discontinuous punctuate pattern. (ii) There was considerable cell-to-cell variability in binding and uptake of GXM. (iii) Thioglycollate-elicited macrophages bound much more GXM than resident peritoneal macrophages. (iv) The accumulation of GXM was blocked by inhibitors of actin and to a less extent by inhibitors of tubulin. (v) The accumulation of GXM was blocked by inhibitors of tyrosine kinase, protein kinase C, and phospholipase C, but not inhibitors of PI3K.

The rate of accumulation of GXM varied with the GXM concentration; a faster rate of accumulation was observed with higher doses. The gradual accumulation of GXM by elicited macrophages resembles the gradual accumulation of GXM by monocyte-derived macrophages that was reported by Monari et al. (75) and was slower than the

very rapid accumulation (minutes) found with neutrophils. There was a maximum level for accumulation that was not exceeded by extended incubation time or by use of higher concentrations of GXM. This threshold effect suggests that potential receptors may have been saturated or depleted.

Although the accumulation of GXM by resting peritoneal macrophages was readily measurable, there was a two-fold increase in GXM accumulation if thioglycollate-elicited macrophages were used. Intraperitoneal injection of thioglycollate broth induces an influx of cells from a pool of preformed monocytes (36). A mixture of exudate cells with resident peritoneal macrophages is consistent with our observation that the population of thioglycollate-elicited macrophages was quite heterogeneous in the uptake of GXM (Fig.3). One of the features of thioglycollate-elicited macrophages is mobilization of C3 receptors for phagocytosis (70). Stimulation with PMA produced a modest but significant enhancement of GXM accumulation by resident peritoneal macrophages (Fig. 4). PMA causes phosphorylation of CD18 (23, 24) and activates CD11b/CD18 (Mac-1, CR3) for phagocytosis (100, 101). Activation of macrophages for increased microbicidal and tumoricidal activity can be accomplished by priming with IFN-γ and triggering with LPS (69). However, such activation produced no increase in GXM uptake by resident peritoneal macrophages. Notably, treatment of resident macrophages with IFN-γ does not stimulate complement receptor-mediated phagocytosis (89).

Drugs that target actin filaments and microtubules were used to assess cytoskeletal requirements for accumulation of GXM. Actin filaments are highly concentrated just beneath the plasma membrane and influence cell shape and locomotion.

In contrast, microtublules determine the positions of membrane-enclosed organelles and direct intracellular transport. Cytochalasin D and latrunculin A inhibit actin function via distinct mechanisms. However, the effects of cytochalasin D and latrunculin A on accumulation of GXM were similar; both agents produced a near complete inhibition of GXM accumulation in a dose-dependent fashion, indicating that uptake occurs via either phagocytosis or pinocytosis. In contrast, microtubule-specific drugs produced a partial inhibition of approximately 50%. One explanation for the partial effect of microtubule-specific agents is the possibility that these drugs do not block GXM attachment but inhibit transport to intracellular depots. This explanation is consistent with the apparent saturation of GXM accumulation that occurred with extended incubation times or use of high GXM doses.

The involvement of cytoskeleton in GXM binding is consistent with the punctuate pattern of GXM binding that was observed on the surfaces of the macrophages. Such punctuate binding suggests the aggregation of potential receptors or binding sites. This aggregation would be made possible by the large, presumably multivalent, nature of the GXM molecule. The affinity and identity of the GXM receptor(s) are not known, but the involvement of multiple receptors would allow for high-avidity interaction. The requirement for cytoskeleton suggests an active process on the part of the cell. Such an active involvement is also suggested by the sensitivity of the process to blockade signal transduction pathways. Notably, one of the consequences of receptor-mediated induction of intracellular signaling can be the targeting of cytoskeletal proteins for altered cell shape or movement.

Tyrosine kinase, protein kinase C, and phospholipase C have roles in several signaling cascades. Our data do not provide information regarding the specific roles of these signaling proteins in GXM accumulation. This is an area that has received very little attention. In this regard, Shoham et al. found that nuclear translocation of NF-κB occurred after GXM stimulation of CHO cells that expressed CD14 and Toll-like receptor 2, human peritoneal blood mononuclear cells, and the murine macrophage cell line RAW264.7 (91). In contrast, when compared with LPS stimulation, GXM stimulation of peritoneal blood mononuclear cells and RAW264.7 cells does not lead to stimulation of mitogen-activated protein kinase pathways leading to phosphorylation of extracellular signal-regulation kinase1/2, stress-activated protein kinase/Jun N-terminal protein kinase, or p38. This report of GXM induced NF-κB translocation coupled with results from the present study suggests that GXM has the potential to stimulate multiple signaling cascades; however, there is much to be learned regarding the intracellular targets and the cellular consequences of such signaling.

Our study used an *in vitro* model for study of GXM uptake by macrophages. The cell-to-cell variability in uptake suggests that there is a subset of macrophages with the ability to accumulate GXM. Several studies have shown that parenteral administration of GXM leads to accumulation of GXM in Kupffer cells of the liver and in the marginal zone macrophages of the spleen (31, 34). In contrast, there was little or no GXM accumulation in lung or brain tissue. Despite the absence of GXM in the lungs or brain, it is possible that GXM could accumulate in alveolar macrophages or microglial cells at the site of infection. Notably, Lipovsky et al. reported that GXM induces interleukin 8 (IL-8)

production by human microglia, raising the possibility of binding and /or uptake of GXM by this biologically relevant cell type (58).

The interaction of GXM with macrophages can impact the pathogenesis of cryptococcosis at several levels. First, ligation of capsular GXM by macrophages can directly mediate uptake of the yeast by macrophages and facilitates complement receptor and $F_C\gamma$ receptor-dependent phagocytosis (82, 92). Second, incubation of phagocytic cells with GXM or encapsulated cryptococci has potent immunoregulatory effects. GXM suppresses LPS-induced secretion of tumor necrosis factor alpha (TNF-α) (94). In contrast, GXM stimulates release of the proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α by human neutrophils (86). Administration of GXM in vivo leads to increased production of macrophage inflammatory protein 1α (MIP-1α), MIP-2, monocyte chemotactic peptide 1 (MCP-1), and IL-1β, IL-6, and TNF-α in the liver (55). Finally, cells of the mononuclear phagocyte system play an active role in the clearance of GXM *in vivo* (34). The results of our study demonstrate that it is possible to experimentally enhance or suppress the binding of GXM to macrophages, raising the possibility for regulation of the interaction between this essential virulence factor and binding sites on cells that are central to host resistance.

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