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MECHANISM OF ACTION OF ANTIBODY TO CAPSULAR POLYSACCHARIDE IN C*RYPTOCOCCUS NEOFORMANS* INFECTION

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1. ABSTRACT

Cryptococcus neoformans is an encapsulated fungus that causes meningoencephalitis in 5-10% of patients with AIDS. While the immune response that controls infection is predominantly cell-mediated, Ab-mediated immunity is being studied for therapeutic use. mAbs to glucuronoxylomannan (GXM), the predominant constituent of the polysaccharide capsule are protective in a variety of murine infection models. However, the mechanism of Ab action in this infection is unknown. We review the literature on the effect of Ab in cryptococcal infection and potential mechanisms of action. The mechanism is likely multifactorial, involving enhancement at several branches of the immune response, including opsonization, antigen presentation and altered effector cell function. Removal of the toxic and immunosuppressive effects of GXM may be an important component of the mechanism of Ab action. Changes in pathology in response to monoclonal antibody (mAb) administration suggest that alterations in cytokine production may mediate mAb effects. In summary, specific Ab can modulate the course of cryptococcal infection to the benefit or detriment of the host, but significant questions remain concerning the mechanism of action and the relative importance of antibody-mediated immunity in normal and immunocompromised hosts.

2. INTRODUCTION

Cryptococcus neoformans is an encapsulated fungus whose most common clinical manifestation is meningoencephalitis, though infection of every organ has been described. Prior to the 1980s, cryptococcal disease was uncommon and occurred predominantly in patients with cell-mediated immune defects. The problem of cryptococcosis has become magnified in the past two decades because it is the most common fungus causing death in patients with AIDS, of whom 6 -10% develop cryptococcal disease (1). This infection is incurable with currently available antifungal drugs, and patients with AIDS who respond to initial therapy must continue lifelong suppressive therapy to avoid relapse (2). Consequently, immunomodulatory therapeutic strategies are being designed to overcome the immune defects that allow disease to develop.

The primary reservoir of *Cryptococcus neoformans* var. *neoformans* is avian excreta, particularly that of pigeons, and humans are believed to acquire the organism by inhalation (3). Although the mechanism for

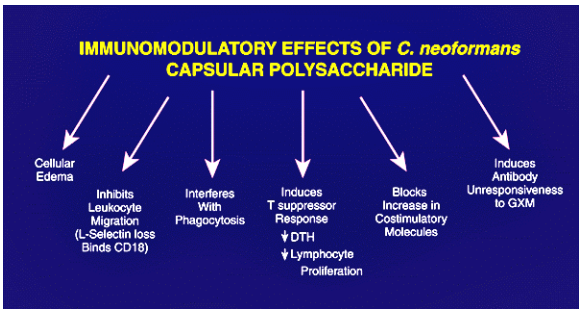

Figure 1. Summary of immunomodulatory effects of cryptococcal capsular polysaccharide.

dissemination is unknown, *C. neoformans* is thought to spread to the central nervous system and to other sites from a primary pulmonary focus. The predominant effective host immune response to infection with this fungus is cell mediated, involving macrophages, $CD4^+$ and $CD8^+$ T cells and NK cells (4-6). However, modulation of antibody-mediated immunity is being studied as a possible mechanism for treatment or prevention of this disease.

The advent of hybridoma technology has allowed the production of monoclonal antibodies to this organism. mAbs to glucuronoxylomannan (GXM), the principal constituent of the capsular polysaccharide, have been shown by several groups to be protective in animal models of infection. One such mAb is being readied for phase I clinical trial. However, the mechanism for antibody-mediated protection remains unknown. We review the literature on the effect of antibody (Ab) in this infection, possible mechanisms for Ab-mediated protection and areas currently undergoing further study.

3. OVERVIEW OF PATHOGENESIS AND THE HOST IMMUNE RESPONSE

In the normal host, initial infection is presumably contained in the lung, while in immunocompromised hosts, the infection can disseminate from a primary pulmonary focus (1,7). Dissemination is presumed to occur hematogenously, but the actual route is unknown, and lymphatic and/or cell-associated spread may occur. That cell-mediated immunity is important for cryptococcal infection can be deduced from the finding that most patients with cryptococcosis have defects in T cell immunity, such as lymphoreticular malignancies, immunosuppression following organ transplantation and HIV infection. One variable in the course of infection is the ability of the host to mount an inflammatory response (see below). In autopsy specimens of brains from patients with AIDS with cryptococcal meningoencephalitis, significant inflammatory responses are not seen, while most non-AIDs patients have granulomatous inflammatory responses (8). *In vitro* lymphocyte function occurs more readily with peripheral blood lymphocytes from patients with positive skin tests than from patients cured of disseminated disease, suggesting that defective lymphocyte transformation may be a factor in the ability of cryptococcal disease to disseminate (9).

C. neoformans may be both an intracellular and an extracellular pathogen, and is found in both locations in tissue. In general, the prevalence of extracellular organisms is inversely proportional to the host inflammatory response. Descriptions of the pathogenesis and pathology of *C. neoformans* ressemble those of *Mycobacterium tuberculosis*, a prototypical intracellular organism (10,11). However, in other respects, the pathogenesis of *C. neoformans* and the Ab response to this organism are similar to those associated with the encapsulated bacteria (recently reviewed in 12). Since the effective host response may require strategies associated with both intra- and extracellular pathogens, *C. neoformans* may present unique challenges to the host immune system.

In murine models, resistance to infection in dependent on the mouse strain (13,14) and the route of infection (15). The early host response that confines cryptococci within the lungs of normal mice infected intratracheally (i.t.) is T cell dependent, and both $CD4^+$ and $CD8^+$ cells are required. While both T cell populations are important in initial containment of infection, $CD4^+$ cells are particularly important in preventing dissemination to the brain (16). T cell transfer from sensitized mice reduces the number of yeast in tissues of infected mice, while B cell depletion and serum transfer play no role (17). $CD4^+$, $CD8^+$ and NK cells have fungistatic activity without the requirement for additional opsonins, while B cells do not (18). A role for B cells in immunity to cryptococcal infection has been demonstrated in some models, though the mechanism by which they exert their effect has not been defined (19).

Macrophages play an essential role in nonspecific cell-mediated immunity to murine cryptococcosis (20). The ability of macrophages to phagocytose cryptococci or to mediate fungistasis or killing depends upon the macrophage source, the state of cellular activation and the presence of opsonins and cytokines. Alveolar macrophages are thought to be the first effector cell type to encounter cryptococci in natural infection (21). The histologic response that contains infection is granulomatous inflammation, in which the major cellular component is the macrophage. The formation of multinucleated giant cells is dependent on the presence of $CD4^+$ cells (16). The association of the development of delayed type hypersensitivity (DTH) with clearance of organisms (15,22) further underscores the importance of macrophages in the host immune response.

4. THE POLYSACCHARIDE CAPSULE

4.1 Immunomodulatory effects

The polysaccharide capsule of *C. neoformans* is required for virulence (23), and is composed predominantly of glucuronoxylomannan (GXM). Other constituents include galactoxylomannan and mannoprotein, which together comprise 12% of the capsule by mass (24). GXM is the major immunodominant antigen (Ag) of encapsulated strains and is the target recognized by most mAbs developed to date. GXM has immunosuppressant effects on multiple arms of the immune response (figure 1). GXM blocks binding of IgG found in normal human serum to the cryptococcal cell wall. Because this Ab binding is required for maximal

rates of attachment to macrophages and for macrophage ingestion of yeast (25), phagocytosis is reduced. Phagocytosis is inhibited by the addition of capsular polysaccharide to acapsular strains *in vitro* (21). Complement activation differs between capsulated and acapsular strain. For unencapsulated *C. neoformans*, classical pathway complement initiation occurs, while for encapsulated strains, complement activation is limited to the alternative pathway (26). The polysaccharide blocks antigenic sites on the cryptococcal cell wall that are responsible for binding of C3 (27). *C. neoformans* capsular polysaccharide (CNPS) also inhibits C5-dependent leukocyte migration (28), blocks IL-8-induced neutrophil chemotaxis (29) and induces shedding of L-selectin by neutrophils, which may in part be responsible for the reduced cellular infiltration into infected tissues of patients with disseminated cryptococcosis (30). GXM also induces a T suppressor cell to secrete a T-suppressor factor via an Ag-presenting cell (31). Cryptococcal culture filtrate antigens induce a T suppressor cascade that results in the suppression of DTH (32,33). CNPS suppresses lymphocyte proliferation *in vitro* (34), and blocks the upregulation of B7-1 expression that occurs in response to coincubation of peripheral blood mononuclear cells (PBMCs) with *C. neoformans* (35). Further, CNPS enhances infectivity of PBMCs for HIV-1 *in vitro* (36) and increases production of p24 Ag after infection of H9 cells with HIV-1-infected H9 cells (37). In mice, injection of CNPS reduces Ab production following challenge immunization with polysaccharide emulsified in Freund's incomplete adjuvant (38). Thus, the cryptococcal capsule downregulates multiple arms of the host immune response in that it is antiphagocytic, prevents the initiation of the classical complement pathway, blocks inflammatory cell recruitment, diminishes Ag presentation in response to infection, suppresses the development of DTH and may reduce Ab production in response to infection. Further, in patients with AIDS, cryptococcal polysaccharide may enhance HIV replication.

4.2 Antigenic characteristics of cryptococcal capsular polysaccharide

Cryptococcal polysaccharide is classified as a T independent Ag because it induces a humoral response in the absence of T cell help and does not induce B cell memory or isotype class switching in secondary immune responses (39). The inability of GXM to induce humoral responses in CBA/cHN *xid* mice and ability to recruit regulatory T cells that both suppress and amplify the specific B cell response further classify it as a Type 2 T independent Ag (40). Cryptococcal strains have been classified into four serotypes on the basis of reciprocal agglutinin absorptions from rabbits immunized with a variety of strains of *C. neoformans* (41-43). By antigenic analysis of the four serotypes by slide agglutination with reciprocal adsorption methods, Ikeda demonstrated the presence of eight antigenic determinants (44).

5. THE AB RESPONSE TO *C. NEOFORMANS*

5.1 Native Ab production

Normal human serum contains Abs that are reactive with cryptococci. IgG from normal human serum can mediate phagocytosis of acapsular cryptococcal strains through an Fc-dependent attachment process by binding to mannoproteins in the cryptococcal cell wall and allowing attachment (45). Though IgG from normal human serum binds to encapsulated strains, it cannot mediate phagocytosis or agglutination because the cell wall-bound IgG is masked and cannot interact with appropriate receptors on the macrophage cell surface (46).

Studies of prevalence of Ab to the capsule or to GXM in patients without cryptococcal disease have yielded varying results. In one study, three percent of sera from normal patients contained antibody that is neutralizable with CNPS (47). Dromer et al reported the prevalence of Ab reactive with capsular polysaccharide to be 20% in patients with AIDS as opposed to 69% of controls, with similar prevalences of IgM and a significantly decreased prevalence of IgG (48). In a study by Houpt et al, among normal subjects, 98% had serum IgM that reacted with GXM, while 28% had IgG (mainly of the IgG2 subclass) and 3% had IgA. The prevalence of IgM Abs was markedly reduced in HIV-infected patients and this reduction occurred in patients with CD4 cell counts $\geq$ 500/ml (49). A more recent study found that IgA, IgG and IgM Abs to GXM are ubiquitous in human sera from both HIV+ and HIV- individuals, and that the IgG present is of the IgG2 subclass, as is found in response to other organisms with polysaccharide capsules (50). However, Abs to an epitope that are present in HIV^- control sera and that are produced in response to the GXM-tetanus toxoid (TT) conjugate vaccine are not found in sera from HIV^+ individuals, suggesting that qualitative differences in the Ab response after HIV infection may play a role in susceptibility to *C. neoformans* infection (51).

The source of GXM-reactive Abs in normal serum is unknown. Their presence may reflect repeated subclinical infection with *C. neoformans* and/or previous exposure to cryptococcal Ags. An equally likely possibility is that these are cross reactive Abs induced by exposure to other organisms, as *Streptococcus pneumoniae*, DF-2 and *Trichosporon beigleii* are antigenically similar to cryptococcal polysaccharide (52-54). Several investigators have reported that naturally occurring Abs are not opsonic for macrophage phagocytosis of *C. neoformans in vitro* (49,55,56).

5.2 The Ab response to infection and vaccination

In response to infection, studies from the pre-HIV era reported the development of serum Ab to *C. neoformans* in 39-79% of patients with cryptococcal disease (57-59). Similar studies have not been repeated in HIV infected patients, though evidence that the Ab response to cryptococcal polysaccharide differs quantitatively and qualitatively in HIV-infected individuals is accumulating (see above, (51). In animal models, Ab production in response to infection is species dependent. Small percentages of both BALB/c and C57Bl/6 mice produce significant titers of IgM and IgG to CNPS in response to infection which decline despite persistence of chronic infection (60,61). Endotracheally infected rats make a transient IgM response, followed by persistent IgG production (62). In early mouse studies in Swiss mice, immunization with whole, killed cells or their products did not elicit protective Ab production (63). Production of agglutinating Abs occurs more frequently in

immunized rabbits than in mice (64). Susceptibility to infection in rabbits, rats and mice parallel their ability to make Ab. Rabbits and rats are high responders and control infection, whereas mice seldom respond and are a highly susceptible species (62,65).

The effect of Ab produced in response to cryptococcal infection is unknown, though in studies from the pre-AIDS era, the absence of serum Ab correlated with treatment failure (57) and the presence of Ab correlated with cure (59). Though most patients from the pre-AIDS era had underlying cellular immune defects, cases of patients with cryptococcosis whose sole defect was hypogammaglobulinemia were reported (66,67). Disseminated cryptococcosis has also been associated with hyper-IgM syndrome (68). In animal models, the benefit to the host of Ab generated in response to vaccination or infection varied. In a rabbit meningitis model in which rabbits are infected intracisternally, IgG that was opsonic for rabbit peritoneal macrophages was produced in the CNS, and the presence of IgG correlated with reduction in CFU (69). However, in other studies, mice immunized with cryptococcal polysaccharide-bovine gamma globulin conjugates developed high levels of Ab to polysaccharide, but the presence of Ab in these mice did not protect them from infection or prolong survival, and the presence of Ab did not correlate with CFU reduction (70). In a study by Monga, B cell-deficient mice did not have altered survival, CFU, DTH or Ag levels following i.v. infection (71). However, Ab titers were not measured in this study, and the absence of altered outcome may reflect the fact that even normal mice seldom make Ab responses during infection. In rabbits infected intracranially in a chronic meningitis model, preimmunization resulting in production of serum Ab did not affect yeast counts, dissemination from the CSF was not prevented and Ab was not measurable in the CSF (72). Differences in therapeutic efficacy of Ab produced in response to vaccination may reflect differences in the isotype or idiotype produced or differences in animal or cryptococcal strains.

6. POLYCLONAL AB IN THERAPY AND ANIMAL STUDIES

Interest in the use of Ab therapy for cryptococcal disease predates the development of antifungal drugs. Prior to the development of mAb technology, heterologous serum was used. In 1925, Shapiro tried to both vaccinate a patient with cryptococcal meningitis and to infuse him intrathecally with rabbit serum from animals inoculated with the patient's cryptococcal isolate. Therapy was discontinued because the patient became sensitized to the rabbit serum (73). Gordon and collaborators treated three patients with combined Amphotericin B and rabbit anticryptococcal Ab in the 1960s, with evidence for serologic response in at least one patient (74). In animal models, the effect of administration of polyclonal Ab preparations varied. Gadebusch showed protection of mice injected with rabbit anticryptococcal serum that was type specific (75). In another study, passive immunization by preincubation of yeast in polyclonal rabbit serum prolonged survival in complement sufficient, but not complement deficient mouse strains infected i.v. (76). Immune rabbit serum increased ingestion of cryptococci by rabbit peritoneal and alveolar macrophages in vitro and by murine neutrophils in vivo (77). However, when begun after infection, injection of rabbit or mouse plasma from animals immunized with cryptococci failed to protect mice infected with an ordinarily lethal inoculum (78). The differences in protection seen with Ab elicited by vaccination or passively administered polyclonal serum may have resulted from differences in epitope specificity or isotypic variances (see below), or from differences in route or timing of administration.

7. MABS TO THE CAPSULE OF *C. NEOFORMANS*

7.1 Description

Since the mid 1980s, mAbs to the cryptococcal capsule have been produced by at least four independent laboratories. Murine mAbs have been produced from infected mice (79), and from mice immunized with GXM-TT (79), purified CNPS (80), and CNPS conjugated to sheep erythrocytes (81). In addition, human mAbs have been generated from volunteers vaccinated with GXM-TT (82) and mouse-human chimeric Abs have been constructed (83). Protective, nonprotective and disease-enhancing mAbs have been described, and mAb epitope and isotype are both important determinants of mAb efficacy (84). Study of these Abs has provided new knowledge about the antibody response to this organism. Comparison of molecular characteristics of mAbs produced in response to infection with a serotype A strain and the GXM-TT conjugate vaccine showed that Ig variable gene usage is highly restricted, as most of the antibodies use the same V_H7183 gene element, have a seven codon CDR3 and utilize J_H2, $V_K5.1$ and J_K1 gene elements (85). Of two IgM mAbs originating from the same B cell that differ only by somatic mutations in the variable regions, one is protective while the other is not (86). Differences in a few amino acid residues, particularly in the heavy chain complementary determining region 2 (CDR2), are associated with variation in fine specificity and protective efficacy of the mAbs (87). Based upon their ability to confer protection, their fine specificity and molecular structure, mAbs to GXM have been classified into six groups (88). Further study of the fine specificity of mAbs developed by hybridoma technology has used phage display peptide libraries to determine peptide binding motifs in antigen binding sites. This technique can potentially identify mimotopes that elicit mAbs to the protective epitopes on the capsular polysaccharide (89).

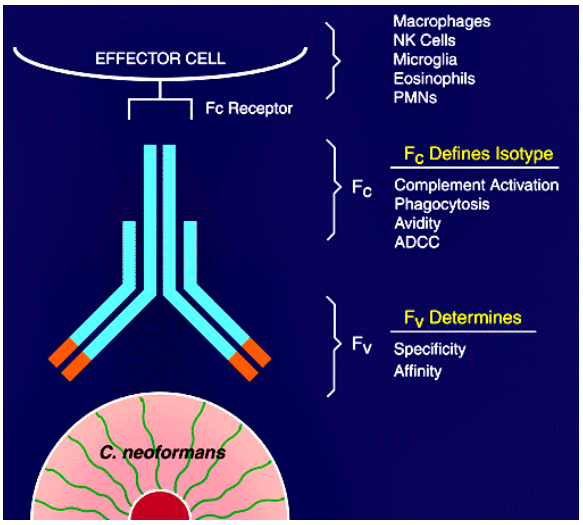

Figure 2. Overview of the roles of Fc regions of Ab in *C. neoformans* infection. Diagram highlights the role of Ab molecules as bridges between effector cells and *C. neoformans* cells. Noted are the roles of Fc and Fv regions in Ab function and cell types associated with Ab-mediated protection are listed.

"Protective" mAbs to GXM prolong survival following i.v., intraperitoneal (i.p.), intracranial (i.c.), and i.t. infection with a variety of *C. neoformans* strains (84,90-93). They also provide additional antifungal benefit when administered in conjunction with several antifungal agents (94-98). Nonprotective and disease-enhancing mAbs are generally defined by their effect on survival relative to saline or to isotype matched control mAbs. While the mAbs are cross-reactive with all serotypes, the ability of mAb to prolong survival and to reduce CFU varies with the cryptococcal strain (99) and, in murine infection models, with the mouse strain (100). Immunofluorescent binding patterns of mAb to the cryptococcal capsule vary, and an annular fluorescence pattern has been associated with a protective response (87).

While mAbs have also been produced to other cryptococcal targets, including galactoxylomannan contained in the capsule and cell wall (101) and to cytoplasmic Ags (102,103), further study of the effects of these mAbs has been limited. The remainder of the discussion is limited to mAbs to GXM.

7.2 The role of isotype

The mAbs to GXM that have been produced are isotype restricted, though mAbs of every isotype have been produced by isotype switching (104). Hybridomas produced from infected mice are predominantly of the IgM class, consistent with the classification of GXM as a T independent Ag, while those produced following immunization are most commonly IgG (85). mAb isotype affects the ability of the mAb to be protective (figure 2). In one family of mAbs derived from the same B cell, IgG1 mAbs prolong survival, reduce tissue CFU and serum GXM in murine infection, while IgG3 mAbs do not. Isotype switching of a disease-enhancing IgG3 resulted in production of a protective IgG1 (105). Studies using knockout mice show that CD4+ cells are required for the protective effect of the IgG1 mAb, but not for the disease enhancing effect of IgG3. IgG3-mediated disease enhancement requires CD8+ cells. In interferon (IFN)-gamma knockout mice, the IgG1 is no longer protective and the IgG3 does not reduce survival (106).

The impact of isotype on the ability of mAb to enhance cryptococcal phagocytosis and killing by macrophages has been studied by several groups with some variance in results. For example, with one family of mAbs derived from the same B cell that have the same idiotype and use identical variable region genes, the relative opsonic ability of the mAbs is IgG1=IgG3>IgG2a>IgG2b>IgM>IgA (107). However, the ability of these mAbs to increase cryptococcal killing by the murine macrophage-like cell line J774.16 is IgG1>IgG2a>IgM>IgG3>IgG2b>IgA (107). Their relative ability to agglutinate cryptococci does not correlate with protective efficacy (84). Another group that produced a family of isotype-switch variants found that the relative ability of their mAbs to opsonize *C. neoformans* for phagocytosis was IgG2a>IgG1>IgG2b (108). However, while with this family, the IgG2a and IgG2b subclasses reproducibly reduced CFU in the lung and spleen in murine i.v. infection, survival was not prolonged by any of the isotypes (109).

Thus, Ab isotype clearly plays a role in the ability of mAbs with the same fine specificities to protect mice in murine infection, as well as to promote opsonization and fungal killing by murine macrophages and macrophage-like cell lines. The reason for differences in protective ability of mAb subclasses is not understood. The explanation for the varying effects of isotype between mAb families is also unknown, but may relate to the fine specificities of the mAbs involved, differences in the cell lines used in these studies or to the cryptococcal strains (107). The importance of isotype is not surprising since isotype mediates many biological effects of Ab, including the ability to fix complement, facilitation of Ab-dependent cellular cytotoxicity (ADCC) and relative avidity. Different isotypes bind to different Fc receptors (FcRs), which may result in initiation of different signal transduction pathways (110). Differences in the isotypes that are best at mediating each of these effects suggest that macrophage cell killing and phagocytosis are not the only mechanism of mAb action. Further, the findings presented above illustrate that isotype-specific effects are dependent not only on FcR binding, but on the presence of other components of the immune response, such as T cell recruitment and cytokine production.

8. POTENTIAL MECHANISMS OF AB ACTION

The mechanism by which mAb administration prolongs survival in animal models is unknown at the present time. Although mAb binding to GXM produces structural alterations in the cryptococcal capsule (111) (illustrated in figure 3), several groups have shown no effect of Ab on fungal growth or viability (100,107,112). Agglutination, a classically described Ab property, may contribute to

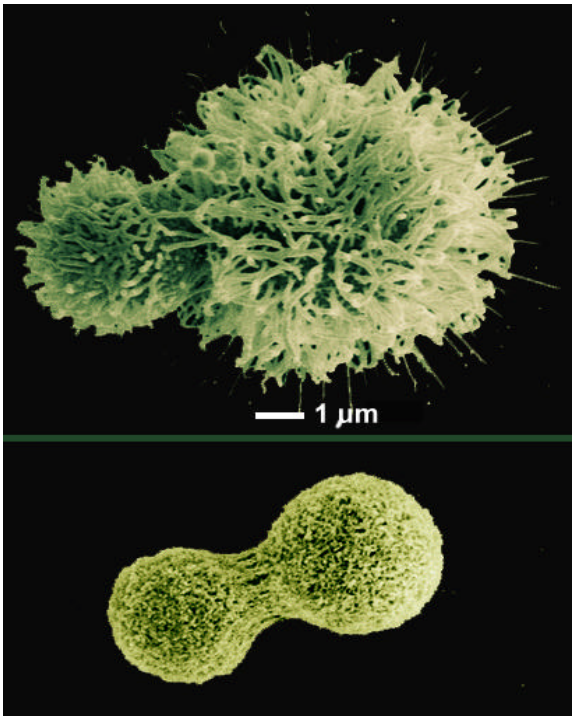

Figure 3. Top: Scanning electron micrograph of encapsulated *C. neoformans* shows fibrillar appearance of the polysaccharide capsule; X 9,000. Bottom: Incubation of yeast in mAb to GXM demonstrates alteration in structural appearance of the polysaccharide capsule; X 8,000. Micrographs were provided by W. Cleare.

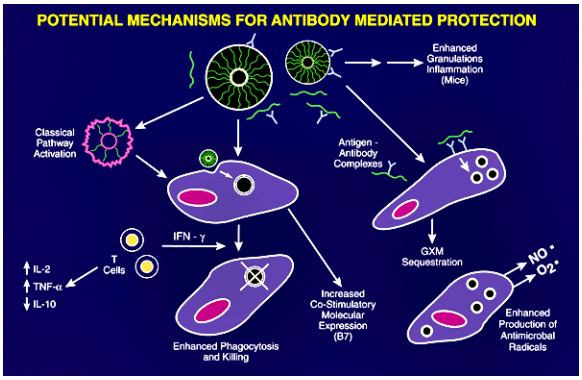

Figure 4. Summary of potential mechanisms of action of mAb in *C. neoformans* infection.

protection in some infections by preventing adhesion or by mechanically clumping organisms and preventing their dissemination. As noted above, the role of agglutination in cryptococcal infection is uncertain, as protective and nonprotective mAbs are agglutinins (84). It is likely that the effects of mAb are multifactorial, and involve actions on macrophages as effector cells, macrophages as antigen presenting cells, altered inflammatory cell recruitment, and differences in cytokine expression. The removal of the toxic and immunomodulatory effects of GXM could provide a common pathway for the mechanism of Ab action. Potential mechanisms of mAb action are summarized in figure 4.

8.1 Opsonization/phagocytosis

The potential mechanism of Ab action in cryptococcal disease that has received the most attention is opsonization for enhanced phagocytosis by macrophages, with the expectation that enhanced phagocytosis results in stimulation of effector cell fungicidal activity. While the requirements for phagocytosis vary based upon the species and tissue from which macrophages are derived and the state of macrophage activation, most commonly, acapsular strains of cryptococcus are readily phagocytosed (113), while encapsulated strains are not efficiently phagocytosed in the absence of opsonins (114-116). In the absence of opsonins, acapsular strains are ingested following binding to mannose and beta-glucan receptors in the yeast cell wall that are blocked by the presence of the capsule (113). While unencapsulated cryptococci are readily ingested by macrophages in the presence of complement, much less ingestion of encapsulated organisms occurs (117). For both encapsulated and acapsular strains, C3b is the major serum opsonin involved in phagocytosis (118), and phagocytosis follows binding to CR1, CR3 and CR4 in human cultured macrophages (55). The cytokines TNF-alpha and GM-CSF enhance phagocytosis by increasing the affinity of CR3 for the yeast (119), and interferon (IFN)-gamma produced by endogenous T cells is required for maximal degrees of phagocytosis and expression of markers of macrophage activation in mice (120).

The ability of polyclonal anticapsular Ab and mAbs to GXM to induce FcR-mediated phagocytosis of encapsulated cryptococci by human and murine macrophages from a variety of tissues and by macrophage-like cell lines is well described, though with some variation in efficacy (55,83,100,107,114,117). However, neither complement receptor- nor Ab-mediated ingestion can be equated with fungal killing, as has been shown in a variety of systems. Absence of fungal killing has been described in guinea pig and human alveolar macrophages where complement was the opsonin (21,114). Intracellular cryptococcal growth is described *in vitro* in human peripheral blood mononuclear cells (121). mAbs to GXM result in Fc-mediated ingestion of cryptococci by microglia, and phagolysosomal fusion is seen. While transient fungistasis follows, some internalized yeast proliferate intracellularly, initiating a chain of events that culminate in cell death (122).

Nonetheless, the ability of Ab to function as an opsonin in the absence of complement is potentially of clinical importance, as complement depletion has been reported in patients and guinea pigs with disseminated cryptococcal disease, but not in patients who are not fungemic (123). Evidence that mAb is beneficial in the role of opsonin in this setting is provided by studies of an i.v. infection model in complement deficient mice. In this model, C5 deficiency results in increased susceptibility to *C. neoformans* (124). Complement deficient ($C5^-$) mice die rapidly from pneumonia, while complement sufficient strains ($C5^+$) die from subacute meningoencephalitis. Soon after infection, PMNs are recruited to the lung in $C5^+$ but not in $C5^-$ mouse strains (125).

The IgG1 mAb E1 prolongs survival of $C5^-$ mice when given prior to infection without restoring the protective PMN infiltration seen in $C5^+$ strains. In the absence of altered inflammatory cell recruitment, survival prolongation and reduction in lung CFU is attributed to enhanced opsonization of cryptococci, with resulting enhancement of macrophage effector function (100).

While increased macrophage killing of cryptococci is likely to result from enhanced phagocytosis, several lines of evidence suggest that this is not the sole mechanism of Ab action. As noted above, the ability of different isotypes to mediate phagocytosis does not completely correlate with enhancement of killing (107). In i.t. models, C5 deficiency is not associated with altered numbers of PMNs recruited to the lung or with decreased fungal clearance (126). Further, in an i.t. murine infection model, cryptococci are phagocytosed rapidly by alveolar macrophages without administration of exogenous Ab (93). While mAb administration prolongs survival in both C5- and C5+ mouse strains, small reductions in lung CFU are seen at most (61,93). However, it is possible that mAb serves different functions in disseminated disease than in initial infection. Further, it is unknown whether the outcomes of FcR- and CR-mediated phagocytosis are the same in cryptococcal infection and this remains an area for further study. Thus, while it is likely that opsonization of cryptococci with increased phagocytosis is one effect of Ab, is unlikely that this is the sole mechanism by which mAb exerts its protective effect.

8.2 Macrophage effector functions (killing)

In many microbial infections, phagocytosis allows more effective killing of microorganisms by the production of antimicrobial peptides, including lysozyme, defensins, cryptidins and histone proteins (127) as well as the production of toxic oxygen and nitrogen radicals (128). Several of these mechanisms may be operative in extracellular killing. Knowledge of the role of these mechanisms in the host response to cryptococcal infection is incomplete, and their relative roles may vary between species and populations of cells. However, it is known that killing of acapsular and encapsulated cryptococci by murine resident peritoneal macrophages is enhanced by stimulation with IFN-gamma (129) and that killing by bone marrow macrophages may occur extracellularly (130).

The effect of Ab to GXM on the ability of macrophages to kill C. neoformans has varied in in vitro systems. As noted above, in in vitro studies, mAb to GXM increases phagocytosis and killing of fungi by the murine macrophage-like cell line J774 (107) and by murine peritoneal and alveolar macrophages (93,100). Diamond reported that in the presence of polyclonal anticapsular Ab, human peripheral blood mononuclear cells can kill C. neoformans extracellularly, by a non-phagocytic mechanism (131). Thus, Ab may mediate anti-cryptococcal activity by effector mechanisms that do not require phagocytosis. However, Levitz found no effect of Ab opsonization on human peripheral monocyte/macrophages, with no killing of an encapsulated strain or an isogenic acapsular mutant (132).

Anticapsular IgG activates murine peritoneal macrophages to synthesize nitrite and to kill cryptococci. In the absence of Ab, stimulation with IFN-gamma is required for fungal killing and both IFN-gamma and serum opsonization are necessary for nitrite production (129). Both anticapsular IgG1 mAb and IFN-gamma are required for stimulation of the respiratory burst of encapsulated yeast, while acapsular organisms stimulate the respiratory burst without a requirement for opsonins. Nitrite synthesis, but not phagocytosis, respiratory burst stimulation or lysosomal enzyme release, correlate with fungal killing in this system (129). Opsonization of encapsulated *C. neoformans* with mAb increases peroxynitrite production and fungicidal activity of IFN-gamma-activated resident peritoneal macrophages and stimulates O_2^- production from unstimulated cells (133).

Naslund found that while acapsular strains induce NOS production by J774 cells as measured by nitrite production, conditions which promote attachment and/or phagocytosis do not lead to NOS induction, including opsonization with specific polyclonal rabbit serum (134). Fc receptor activation by GXM-mAb complexes enhances production of nitrogen related oxidants by IFN-gamma-stimulated J774 cells (135). However, IgG1-mediated killing of *C. neoformans* by J774 cells occurs despite inhibition of NOS and ROI scavengers (136). The specific mediators of this fungal killing are unknown. Similarly, the transient fungistasis following incubation of *C. neoformans* with fetal human microglial cells in the presence of an IgG1 mAb is not inhibited by NOS inhibitors and ROI scavengers (137).

In vivo, administration of mAb to the polysaccharide capsule reduces fungal burden in a variety of murine models of infection. It reduces lung CFU following i.v. infection with a variety of *C. neoformans* strains and reduces brain CFU following i.c. but not i.v. infection (90-92). In i.t. infection, despite marked prolongation of survival, CFU reductions in the lung are small and there is no reduction in extrapulmonary sites (93). Increased fungal killing by macrophages may contribute to the beneficial effect of Ab, but the lack of association of survival prolongation with reductions in fungal burden suggest that this is not the major mechanism of Ab action in i.t. infection.

8.3 Granulocytes as effector cells

Comparatively little attention has focused on the role of neutrophils and eosinophils in Ab-mediated protection against *C. neoformans*. Both cell populations are present transiently after infection in animal models, though their importance in the immune response in human infection is unknown. In i.v. infection, neutrophils are present in the pulmonary vasculature of murine lung, where ingestion is dependent on the presence of C5 and terminal complement components. Though prominent among intravascular inflammatory cells at 30 min after infection, they are rare by 24 h. While after i.t. infection no role for these mechanisms has been seen (126), neutrophils are present in pulmonary infiltrates during the first 7 d, but not subsequently (138). Neutrophils are the first inflammatory cells to migrate toward

cryptococci in the peritonea of rabbits infected i.p., and are then replaced by monocytes (65). *In vitro* phagocytosis of cryptococci by human peripheral blood PMNs is dependent mainly on the presence of components of the alternate complement pathway (112). Neutrophil activation enhances phagocytosis of cryptococci opsonized with normal human serum, and this enhancement may be related to increased CR3 expression (139). Once ingestion occurs, killing of yeast is relatively efficient, and myeloperoxidase and H_2O_2 are necessary for killing (112). Incubation of human peripheral blood neutrophils with *C. neoformans* or with GXM results in production of the proinflammatory cytokines TNF-alpha, IL-8, IL-1beta and IL-6 (140).

Results of *in vitro* studies using polyclonal sera vary. While one study concluded that Ab to *C. neoformans* does not significantly increase ingestion or killing by neutrophils *in vitro* (112), another showed that anticapsular Ab is required for maximal phagocytosis by these cells (141). A third demonstrated that Ab is required for neutrophil-mediated killing, and that in the presence of antiserum, neutrophil killing of *C. neoformans* is more efficient than that by monocytes (142). A recent study showed that a human anti-GXM IgM increases neutrophil phagocytosis and growth inhibition of cryptococci compared to complement alone, suggesting that Ab-mediated deposition of complement components on the capsule can enhance neutrophil complement receptor-mediated antifungal activity (143).

Eosinophils are prominant in pulmonary inflammatory cells 14 d after i.t. infection in some mouse strains, but then gradually decrease in number (144). Eosinophils are uncommonly reported in inflammatory responses to human infection. Phagocytosis of cryptococci by rat peritoneal eosinophils is induced by IgG1 and IgE to GXM *in vitro*, and degranulation is seen. Minimal phagocytosis occurs in the absence of Ab (144). *In vivo*, administration of IgG1 results in reduction in the number of granules per eosinophil in murine lung, suggesting Fc-mediated degranulation, and in occasional eosinophil phagocytosis of cryptococci (61). The effect of eosinophil phagocytosis or degranulation on cryptococcal killing by yeast is unknown, though the lack of reduction in lung CFU seen in mAb-treated mice in this model suggests that this mechanism is unlikely to be a major contributor to Ab-mediated effects in mouse lungs.

Both neutrophils and eosinophils may have important roles in the host immune response to this pathogen. Their transient appearance after initial infection may account for their lack of prominence in human pathology specimens of *C. neoformans* infection, which are usually from patients with longstanding infection. Intravascular neutrophil phagocytosis of cryptococci suggests that these cells may be important for prevention of intravascular dissemination. Both cells can function as effector cells through multiple mechanisms, including target killing and cytokine secretion. Ab may potentially act by enhancing these roles. Further study is required for determination of the roles of these cells both in the immune response to infection as well as in Ab-mediated protection.

8.4 Antigen presentation

A mechanism of Ab action in cryptococcal disease currently under investigation is enhancement of antigen presentation. To date, all studies of this effect have been done *in vitro*. In cryptococcal infection, lymph node cells primed with acapsular organisms proliferate in response to acapsular organisms but not to encapsulated yeast. Thirty fold more encapsulated yeast are required to induce such a response. Therefore, inhibition of antigen presentation is another deleterious effect of cryptococcal capsular polysaccharide. However, once ingestion of organisms occurs, the capsule has no effect on processing or presentation of cryptococcal antigens and subsequent T cell activation (145). Vecchiarelli has shown that normal human serum allows phagocytosis of thinly encapsulated cryptococci by human alveolar macrophages, but that phagolysosomal fusion is inhibited. Co-culture of alveolar macrophages with autologous T cells produces a massive blastogenic response of alpha/beta TCR-bearing T cells that is regulated by IL-1 produced by the macrophages in response to cryptococci (146). Thus, macrophages may be important Ag presenters in cryptococcal disease.

Addition of the IgG1 mAb 2H1 to cocultures of cryptococcal-laden monocytes plus autologous T cells increases lymphoproliferation, and the magnitude of effect is dependent on mAb concentration (147). This alteration is associated with a reduction of IL-10 found in the culture supernatant, and removal of IL-10-induced downregulation of MHC class II expression may be responsible for the increased lymphoproliferation (147). mAb 2H1 increases B7-1 expression on peripheral blood mononuclear cells in the presence of encapsulated strains of *C. neoformans*, but not in the presence of acapsular strains or in the absence of *C. neoformans* (35). Thus, the capsular polysaccharide may interfere with antigen presentation by downregulation of MHC class II expression in an IL-10 dependent process, and may prevent the upregulation of expression of co-stimulatory molecules. *In vitro* studies suggest that Ab may reverse these deleterious effects and allow more effective inflammation. *In vivo* studies will further define this possible mechanism.

8.5 Antibody-dependent cell-mediated cytotoxicity (ADCC)

NK cells are the principal mediators of this nonspecific arm of the immune system, in which NK cells develop cytolytic capacities without need for prior contact with Ag through binding of aggregated IgG to FcgammaRIII. In the absence of exogenous Ab, NK cells are responsible for growth inhibition of *C. neoformans* in nonadherent spleen cells *in vitro* (148) but treatment with anti-asialoGM-1 Ab to decrease NK activity does not affect survival in a murine i.v. model of infection (6). The importance of this mechanism in native infection is therefore unknown.

However, human NK cells function as effectors in ADCC only in the presence of rabbit anticryptococcal antiserum (149). *In vitro*, polyclonal rabbit anti-cryptococcal IgG accelerates the anticryptococcal effects of NK cells, which mediate their activity by extracellular killing through binding to the cryptococcal cell wall (150). Thus, a potential mechanism of Ab action is the enhancement of NK cell effector activity. *In vivo*, beige mice, which have defective NK

cells that are unable to lyse NK-sensitive targets, have increased susceptibility to *C. neoformans* infection (151). In this mouse strain, the effects of mAb are similar to those seen in immunocompetent mice after i.v. infection, raising question as to the role of NK cells in Ab-mediated protection (106). Demonstration of the role of Ab in this regard requires further study.

8.6 Removal of toxin (GXM)

As noted in section 4.1 above, GXM has many immunomodulatory effects. Hence, the reversal of GXM-induced immunosuppression is likely to contribute to the action of mAb in cryptococcal infection. Removal of GXM by Ag-Ab complex formation potentially reverses these deleterious actions on the immune system and may also reverse the direct toxicity of polysaccharide in tissue. In human autopsy specimens from patients who died from cryptococcal meningoencephalitis, CNPS is detectable by immunohistochemistry in the brain parenchyma and meninges. Higher percentages of tissue cross sectional area are involved in specimens from patients with AIDS than from those without AIDS (8). Capsular polysaccharide spreads through CNS tissue after intracranial inoculation in rats, resulting in cell swelling (152,153). In murine i.v. infection models, CNPS is shed into tissue, and administration of mAb results in removal of immunohistochemical staining for GXM in areas away from fungi in the brain. In the lung, polysaccharide lines the alveolar epithelium and bronchial lumen, while in mAb-treated mice, staining is limited to granulomas (90). mAb administration results in reduction in serum GXM in mice infected by i.p., i.v., and i.t. routes (84,90,93) and mAb effectively removes GXM from rats inoculated i.v. (154). mAb-treated mice have lower brain weights despite no reduction in CFUs. This finding may reflect reduced brain edema and suggests a "mechanical" protective mechanism (90,92). Protective efficacy of mAbs administered after established infection does not correlate with reduction in serum GXM (94). Results of studies with polyclonal IgG show that cryptococcal Ag-Ab complexes may exert detrimental effects by preventing macrophages from ingesting immunologically coated cryptococci via their FcRs (155). Nonetheless, the reversal of GXM-induced immunosuppression and the removal of direct toxic effects are likely to be important components of the action of mAb in cryptococcal disease.

8.7 Altered pathology

Containment of cryptococci is associated with the development of granuloma formation while in disseminated disease, absence of inflammation is characteristic. Though the mechanism of granuloma formation in cryptococcal infection is not well understood, one potential mechanism of Ab action is the enablement of a more "effective" pattern of inflammation. Early ultrastructural studies describe granuloma initiation in the peritonea of rabbits and *in vitro* with rabbit and guinea pig peritoneal cells. While small yeast are phagocytosed by PMNs or monocytes, larger fully encapsulated organisms are first surrounded by rings of PMNs that are later substituted by monocytic rings. These rings then fuse into giant cells, with release of hydrolytic enzymes (156,157). The formation of multinucleated giant cells is dependent on the presence of $CD4^+$ cells (16).

Early studies by Aronson, et al., and Schneerson-Porat et al, show differences in the inflammatory response of mice infected i.p. when cryptococci are incubated in immune rabbit serum. Mouse monocytes require addition of immune serum for phagocytosis of cryptococci and ring formation. When yeast are incubated in Ab, mononuclear cells adhere to cryptococci and cryptococci agglutinate. Ab opsonization results in the formation of three dimensional structures in which yeast are surrounded by mononuclear cells which then fuse (158,159). In rabbits and guinea pigs, two species that are less susceptible to infection, immune rabbit serum is not required for ring formation, though organisms are only completely enclosed in the presence of Ab opsonins. In rabbits, serum is not required for ring formation, but plasma cells are seen in the inflammatory infiltrate (65). These studies suggest that Ab to the capsular polysaccharide alters the inflammatory response that mononuclear cells can form.

In mice infected i.t., prolongation of survival by administration of an IgG1 mAb to GXM is associated with alteration in lung pathology such that yeast are contained within foci of inflammation, while in control mice cryptococci spread through the alveolar spaces (93). These studies suggest that Ab can result in more effective containment of cryptococci within granulomas. Since granuloma formation, which is analogous to chronic DTH, results from products of macrophage activation, such as cytokines, a possible mechanism for the pathology differences seen in the presence of Ab is alteration of cytokine production by host inflammatory cells.

8.8 Inconsistencies

While some or all of the mechanisms described above may contribute to the mechanism of mAb action against *C. neoformans*, inconsistencies in the effects of mAbs in *in vitro* and *in vivo* systems demonstrate that our knowledge of mAb action is far from complete. For example, IgG3 are non-protective, yet are opsonic, promote fungal killing *in vitro* by macrophages and clear serum polysaccharide (94,107). IgG1 are protective in beige mice that have no functional NK cells (106). These results suggest that care must be taken in the extrapolation of *in vitro* results. Further, the discrepancies indicate that the *in vivo* effects may be the cumulative result of multiple mechanisms, all of which are not presently known.

Furthermore, it is uncertain why Ab administration fails to clear the infection. Possibilities include a problem with the models used, an inherent limitation to Ab-mediated protective mechanisms, active interference with Ab-mediated protection by the fungus and/or a combination of the above.

8.9 New directions: Altered cytokine expression/cellular recruitment

Recent study has shown that resistance to cryptococcal infection and development of protective inflammatory responses are associated with the production of the T_H1-associated cytokines (14), as is typical for diseases in which granulomatous inflammation is responsible for

containment. In this regard, IFN-gamma and IL-12 are required for inflammatory cell recruitment in the lung following murine i.t. cryptococcal infection. IFN-gamma is required for containment of yeast within inflammatory foci (160), while IL-12 suppresses dissemination (161). Administration of anti-TNF-alpha mAb reduces inflammatory cell recruitment, increases CFU and prevents the development of DTH (162). The inflammatory changes produced by these cytokines parallel the histopathological changes seen following mAb administration in susceptible mice, as described above. Limited studies of the ability of mAb administration to alter cytokine production *in vivo* have been performed to date. However, preliminary data suggests that an effect of mAb in murine pulmonary infection may be to alter cytokine production, possibly through reduction of T_H2-associated cytokines (163).

In *in vitro* studies, during coculture of human alveolar macrophages with T cells and cryptococci, following a lymphoblastogenic response, high levels of IFN-gamma and IL-2 are found in culture supernatants, while IL-4 is undetectable. Levels of IFN-gamma and IL-2 are higher in response to incubation with acapsular than with encapsulated strains (164). IL-10 production causes dose-dependent inhibition of TNF-alpha and IL-1beta release by peripheral blood mononuclear cells in response to *C. neoformans* and reduces mRNA expression for TNF-alpha (165). PBMs produce higher levels of IL-10 in response to encapsulated cryptococcal strains than acapsular strains (166). A mechanism of Ab action in cryptococcal infection may be reduction of IL-10 production, removing the inhibitory effect on proinflammatory cytokine production (147).

Alteration in cellular recruitment at the chemokine level as opposed to at the effector cytokine level is another possible pathway for the histopathological changes that accompany mAb administration. Additional pathways for cellular recruitment in cryptococcal infection are newly being studied. Recently, Huffnagle et al reported that MIP-1alpha, which is chemotactic for a variety of inflammatory cells, selectively recruits macrophage/monocytes and PMNs to the lungs of mice infected with *C. neoformans* and that depletion of this chemokine is associated with reduced fungal clearance. Induction of MIP-1alpha secretion is dependent on MCP-1 production (167). There is no data on the ability of mAb to GXM to produce changes in these chemokines. However, this is a potential area for further exploration.

9. PERSPECTIVE

The role of Ab immunity in cryptococcal infection and the mechanism by which Ab to the cryptococcal capsule protects in experimental infection remain unknown. The advent of mAb technology has allowed the generation of reagents which have been useful for dissection of the Ab response to this pathogen and for study of the effect of Ab on the host response to infection. Macrophages remain prime suspects for the cells on which mAb acts. Recently, however, attention has focused beyond the standard Ab functions of opsonophagocytosis resulting in enhanced killing toward exploration of areas such as enhancement of macrophage function in antigen presentation. Further, the complex nature of the development of granulomatous inflammation that allows containment of infection, and the apparent ability of Ab to the capsular polysaccharide to enhance this containment in murine infection has turned attention toward possible differences in cytokine milieu that may follow Ab administration. Though study of the effects of Ab on cytokines is likely to yield a heterogeneous body of information, with potential effects on a number of aspects of the immune response, differences in the setting of Ab administration will likely point to new mechanisms by which Ab exerts its effects.

The study of Ab immunity in cryptococcal infection has produced observations that are unparalleled in Ab-mediated protection against other pathogens. The phenomena concerning IgG1 and IgG3 have not been described in microbial immunity for other pathogens. The dependency of Ab-mediated immunity on T cell function is also remarkable. These observations may reflect uniqueness of this encapsulated pathogen that is both intra- and extracellular and requires granuloma formation for containment. Further, work on *C. neoformans* has contributed to re-evaluation of the role of Ab against other pathogens for which classical Ab immunity is not thought to be important, such as *Candida albicans* (168) and *Mycobacterium tuberculosis* (169).

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