Towards a vaccine for Cryptococcus neoformans: principles and caveats

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Abstract

In the Damage-response framework of microbial pathogenesis, infectious diseases are one outcome of a host-microorganism interaction in a susceptible host. In cryptococcal disease, damage to the host is caused by Cryptococcus neoformans virulence determinants, the nature of the host response, or both. Further, the disease may be acute or reactivated from a latent state. Hence, a vaccine for C. neoformans would need to prevent disease resulting from either acute or reactivated infection. The evidence to support the development of a vaccine for C. neoformans that induces antibody-mediated immunity is discussed herein.

Introduction

In around the last hundred years, our capability to diagnose, treat and prevent various infectious diseases has been markedly improved through advances in medical research and therapy, including the introduction of antimicrobial chemotherapeutic agents. Paradoxically, however, there has been an unprecedented increase in the number of patients that are vulnerable to a wide array of infectious diseases. The responsibility for this rests, in part, on the introduction and therapeutic usage of anti-neoplastic chemotherapy and organ and bone-marrow/stem cell transplantation to treat malignancies, which result in or require suppression of normal immunity. In addition, the advent of intensive-care units and widespread use of intravascular catheters and broad spectrum antibacterials has led to the growth of a population of patients with impaired barrier immunity. These individuals are immunosuppressed by virtue of having disrupted integument, mucosal surfaces and/or replacement of commensal microbiota with hospital-acquired microorganisms that are more resistant to antibiotics. Further contributing to host vulnerability has been, since the early 1980s, the HIV pandemic. HIV infection has profoundly affected the global population regardless of age, sex, ethnicity and geographical barriers, resulting in large numbers of immunocompromised individuals.

The immunocompromised host is an individual with impaired immunity, including acquired immunodeficiency. Such individuals are at increased risk for many infectious diseases, depending on the nature of their immune defects. The relatively sudden and increasing occurrence of infectious diseases in immunocompromised hosts caused by microorganisms formerly considered to be non-pathogens and/or those rarely seen in individuals with normal immunity provides clear evidence that infectious diseases are but one outcome of host–pathogen interaction, and that they can only occur in a susceptible host (Casadevall & Pirofski, 1999). This concept was put forth in the Damage-response framework of microbial pathogenesis. The Damage-response framework regards host damage as the common denominator of any host-microorganism interaction. However, damage that occurs during host-microorganism interaction reflects a complex interplay of microorganism-induced damage, host-induced damage, or both (Casadevall & Pirofski, 1999). The pathogen classification system proposed by the Damage-response framework plots the degree of host damage as a function of the host immune response, which makes it possible to dispense with terms such as pathogen, non-
pathogen and opportunistic pathogen, and to characterize any microorganism within a unified theory of microbial pathogenesis. The use of this schema allows discrimination of pathogens that cause damage to hosts with normal or weak immune responses from those that cause damage to hosts with weak, as well as strong (excessive) immune responses. By focusing on host damage as an outcome of host–pathogen interactions, it is possible to approach rational vaccine design by identifying targets that can minimize or repair host damage, while avoiding the production of inflammatory mediators, which, despite reducing the microbial burden, could have a detrimental effect.

This article will discuss the prospects for developing a vaccine for Cryptococcus neoformans from the vantage point of how such a vaccine might work by inducing antibody-mediated immunity. Although this article will focus on antibody-mediated immunity, we would like to remind the reader that vaccines that could induce cell-mediated immunity are also under development, and that it is likely that a successful vaccine could promote collaboration between antibody- and cell-mediated immunity.

**Challenges to designing a vaccine for cryptococcal disease**

The pathogen classes, as defined by the Damage-response framework, depict the relative ability of a pathogen to cause damage based on the host immune response. *Cryptococcus neoformans*, along with other microorganisms that possess a polysaccharide capsule and induce similar types of clinical syndromes (*Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitidis*), were originally put in Class 2, because they were not thought to induce damage in the setting of a strong immune response (Casadevall & Pirofski, 1999, 2003b). However, recent studies have revealed that *C. neoformans* can induce an exuberant inflammatory response that results in disease in HIV-infected patients on highly active anti-retroviral therapy (HAART) (Jenny-Avital & Abadi, 2002) and in solid organ transplant recipients after immune reconstitution (Singh et al., 2005). Hence, damage in the setting of *C. neoformans* infection is associated with either a weak immune response, such as in HIV-infected individuals with T-cell deficiency, or an exuberant inflammatory response, such as in HIV-infected individuals or solid organ transplant recipients after reconstitution. The major mechanism of damage in the setting of weak immune responses is often microorganism-mediated. For *C. neoformans*, the microbial component is most likely the capsular polysaccharide, of which the glucuronoxymannan (GXM) constituent has a wide array of deleterious effects (Casadevall & Pirofski, 2005). In contrast, the major mechanism of damage in the setting of a strong immune response is likely to be host-mediated, such as by immune complexes and/or Th1-type inflammatory responses. In light of the discovery that cryptococcosis can occur after immune-reconstitution, *C. neoformans* may be more appropriately classified as a Class 4 pathogen (Fig. 1).

**Does a vaccine for *C. neoformans* make sense?**

The interaction between the human host and *C. neoformans* is likely to begin in childhood (Spitzer et al., 1993; Goldman et al., 2001). Infection is believed to be asymptomatic and the majority of cases of cryptococcal disease occur in adults with underlying immune defects. Several lines of evidence suggest that the outcome of *C. neoformans* infection is a latent state, and that the occurrence of disease represents reactivation (Spitzer et al., 1993; García-Hermoso et al., 1999). Hence, a vaccine for *C. neoformans* would have to prevent reactivation and/or be effective in the setting of established disease. Such a vaccine could be considered a therapeutic vaccine. At present, only the rabies vaccine, which is used after infection in conjunction with antibody, is used as a therapeutic vaccine. However, the success of a varicella-zoster vaccine in preventing herpes zoster in older adults (Oxman et al., 2005) provides proof of the principle that a vaccine can prevent an infectious disease caused by
reactivation of latent microorganisms and raises confidence that a similar vaccine for *C. neoformans* is feasible. Cryptococcal disease can also reflect the outcome of acute infection (Nosanchuk et al., 2000). Therefore, the challenge of developing a vaccine for *C. neoformans* is that, ideally, it should prevent reactivation as well as acute disease. As cryptococcal disease can occur in the setting of both weak and strong immune responses, a vaccine that enhances the immune response could be deleterious by enhancing strong responses, but a vaccine that dampens strong responses could be deleterious by being unable to stimulate weak responses. As such, the present understanding of *C. neoformans* pathogenesis suggests that different vaccine antigens could be required depending on the immune status of the individual to be vaccinated.

The human antibody response to natural cryptococcal infection

Although a defined role for antibody-mediated immunity (AMI) in resistance or susceptibility to human cryptococcosis has not been identified, available data suggest that quantitative and qualitative differences in the antibody repertoires of individuals who are at high risk, compared to those who are not, could translate into an additional risk factor for disease. Available data suggest that antibody deficiency and/or repertoire defects, along with impaired cellular immune mechanisms, could lead to a higher fungal burden and/or host damage from GXM and other cryptococcal virulence factors. The vast majority of patients who develop cryptococcal disease have impaired immunity, most notably HIV-associated T-cell immunodeficiency. The central importance of intact CD4+ T-cell mediated immunity in resistance to cryptococcosis is incontrovertible. However, susceptibility to HIV-associated cryptococcosis must depend on additional factors, as the incidence of disease is markedly less than that of HIV-induced CD4+ T-cell deficiency (Currie & Casadevall, 1994). Further, the ubiquity of *C. neoformans* in the environment and strong evidence for latency in humans (Spitzer et al., 1993; García-Hermoso et al., 1999) suggest that *de novo* infection alone does not cause sufficient damage to result in disease. Most importantly, although *C. neoformans* can exploit host and/or environmental factors to enhance virulence, this cannot explain why only some HIV-infected individuals develop disease, whereas others with similar immunological profiles do not, or why certain healthy individuals develop disease (Casadevall & Perfect, 1998; Banerjee et al., 2001).

The dogma in cryptococcal research has been that AMI is not important for natural resistance to cryptococcosis, particularly as GXM is a poor immunogen and is believed to suppress the immune response (Casadevall et al., 2002; Casadevall & Pirofski, 2005). However, several lines of evidence suggest a more prominent role for AMI in host defense against cryptococcosis. First, immunocompetent individuals are highly resistant to cryptococcosis, and most, if not all, have antibodies to GXM in their serum (discussed in Casadevall & Pirofski, 2005). Patients with immunoglobulin defects, such as hypogammaglobulinemia and X-linked hyper-IgM syndromes, are at an increased risk for cryptococcosis; although these syndromes are characterized by defects in acquired, T-cell dependent antibody responses, they also feature central defects in the B-cell repertoire (discussed in Subramaniam et al., 2005). Similarly, in the pre-AIDS era, patients with the highest risk for cryptococcosis often had B-cell defects in addition to impaired cellular immunity, such as those with HIV-infection, B-cell or hematologic malignancies. Second, at least four independent groups have shown that passive administration of antibodies to GXM or active vaccination with cryptococcal antigens or mimetics can protect mice from a lethal cryptococcal challenge (see Fleuridor et al., 2001; Casadevall et al., 2002; Maitta et al., 2004c). Third, HIV-infected individuals, who are at high risk for cryptococcal disease, have been shown to have quantitative and qualitative differences in their non-specific and GXM-reactive antibody repertoires compared to HIV-uninfected individuals, who are at very low risk for disease (Fleuridor et al., 1999; Subramaniam et al., 2005).

Isotype expression of naturally occurring human GXM-reactive antibodies

GXM- or capsular polysaccharide-reactive antibodies have been identified in human sera by a number of different investigators (Dromer et al., 1988a; Houpt et al., 1994; DeShaw & Pirofski, 1995; Fleuridor et al., 1999; Goldman et al., 2001; Subramaniam et al., 2005). Subramaniam et al. found that HIV-infected African subjects had significantly lower levels of GXM-reactive IgM, but higher levels of total IgM than did HIV-uninfected subjects (Subramaniam et al., 2005). As HIV-associated hypergammaglobulinemia is characterized by an expanded population of naive and a reduced population of memory B cells (discussed in Subramaniam et al., 2005), this could reflect HIV-associated perturbations in the memory B cell repertoire (De et al., 2001; Chong et al., 2004). Reductions in the IgM memory compartment have been implicated in susceptibility to *S. pneumoniae* (Kruetzmann et al., 2003). Naturally occurring IgM is required for resistance to a number of pathogens (Brown et al., 2002; Alugupalli et al., 2003; Diamond et al., 2003). Hence, certain subsets of specific IgM could contribute to resistance to *C. neoformans*. In support of this possibility, human GXM-reactive IgM mediated protection against experimental cryptococcosis (Fleuridor et al., 1998; Maitta et al., 2004a, c). Clinical history influenced the level of GXM-reactive
antibody in HIV-infected Africans (Subramaniam et al., 2005). HIV-infected Africans with a history of pneumonia had higher, whereas those with a history of herpes zoster had lower, levels of GXM-reactive antibodies. These findings suggest that variability in clinical history could be one factor contributing to the difficulty in identifying disease-specific antibody profiles in patients with cryptococcal disease.

Fleuridor et al. (1999) showed that GXM-reactive IgG levels increased among HIV-infected subjects as CD4⁺ T-cell counts declined. Together with data from other studies showing higher levels of GXM- or C. neoformans protein-reactive IgG among HIV-infected subjects (DeShaw & Pirofski, 1995; Chen et al., 1999; Subramaniam et al., 2005), these observations suggest such antibodies could have been elicited by a new infection and/or the reactivation of C. neoformans. Further, high levels of GXM-reactive IgG could be disease-enhancing, perhaps as a clinical correlate of the prozone-like phenomenon observed in mouse models, whereby high amounts of antibody abrogate successful AMI (Taborda & Casadevall, 2001; Taborda et al., 2003; Maitta et al., 2004a). Along the same lines, the apparent inefficacy of AMI in HIV-infected individuals resembles the finding that GXM induces protective and non-protective antibodies in mice, and that the efficacy of AMI can depend on intact cellular immunity (Casadevall & Pirofski, 2005). Antifungals and/or immune reconstitution may reduce the fungal burden in patients with cryptococcosis who are on HAART or recovering from organ transplantation. However, the presence of non-protective and/or high antibody levels could render AMI ineffective and enhance detrimental Th1-type, cell-mediated inflammation. Further work is required to determine whether, and if so, which, human antibody types depend on intact cellular immunity for their biological activity, and whether such antibodies are effective or ineffective in the setting of immunodeficiency and/or reconstitution-associated cryptococcosis.

**Idiotype expression of naturally occurring human GXM-reactive antibodies**

The predominant human immunoglobulin gene family used in antibodies to GXM and other capsular polysaccharides is V₁₃ (Pirofski, 2001), which comprises about half of the circulating V₁₃ repertoire in adult peripheral B cells (Chang et al., 2004). Fleuridor et al. found that HIV-infected subjects who subsequently developed cryptococcosis had lower levels of antibodies expressing V₁₃ than did those who did not (Fleuridor et al., 1999). The finding of decreased V₁₃ expression among HIV-infected subjects (Fleuridor et al., 1999; Chang et al., 2004) was consistent with the phenomenon of HIV-associated depletion of B cells and antibodies expressing V₁₃, which is thought to be mediated by binding of HIV gp120 to variable region epitopes of V₁₃-positive gene products (Chang et al., 2000). In contrast to studies by Fleuridor et al. (1999) and Abadi et al. (1998), which examined cohorts in the United States, Subramaniam et al. (2005) found that V₁₃ antibody levels were elevated in HIV-infected Africans; however, the level depended on clinical history. Among those who developed cryptococcal disease there was a trend towards higher V₁₃ levels. In contrast, those who developed cryptococcal disease, but did not have a history of pneumonia, had a trend towards lower V₁₃ levels. This finding was similar to the finding that HIV-infected children with a history of invasive pneumococcal disease had increased B-cell IgG V₁₃ expression (Chang et al., 2004). GXM-reactive antibodies from African subjects did not manifest substantial cross-reactivity with pneumococcal capsular polysaccharides. Hence, the Subramaniam study raised the intriguing possibility that the African subjects could have had cryptococcal, rather than what was believed to be pneumococcal, pneumonia. More information is required to answer this question. Nonetheless, the fact that a human V₁₃-positive GXM-reactive MAb that was protective against experimental cryptococcosis was ineffective when too much or too little MAb was administered (Taborda & Casadevall, 2001; Maitta et al., 2004a) suggests that altered levels of V₁₃ antibodies could influence susceptibility and resistance to HIV-associated cryptococcal disease.

**The rationale for a vaccine for C. neoformans**

Of relevance to a vaccine for C. neoformans is that many effective vaccines do not prevent infection. The central mechanism by which vaccines mediate protection is by preventing disease. While the precise mechanisms of vaccine efficacy remain to be determined for most available vaccines, their efficacy depends on inducing immune responses that can reduce the host damage that results from the host-microorganism interaction that occurs after an infection below the threshold for disease. This shifts the basic Damage-response curve (Fig. 1) to a point at which there is less host damage. Currently licensed vaccines depend on AMI to prevent disease. A role for AMI against C. neoformans was initially difficult to establish because of limitations in available antibody reagents and animal models; however, the advent and development of MAbs to C. neoformans antigens led to studies that established that AMI could be effective against cryptococcal disease (Casadevall & Pirofski, 2005). It was rapidly recognized that mechanisms of antibody efficacy against C. neoformans included classical antibody functions as well as novel and unexpected functions ranging from direct antimicrobial effects to counteracting cryptococcal virulence factors to modulation of the host inflammatory response (Casadevall & Pirofski, 2004, 2005).
Many lines of evidence support the feasibility of a vaccine for *C. neoformans* that would work by inducing AMI. Antibodies to GXM have a variety of functions and mechanisms of action, including that they promote phagocytosis, alter the inflammatory response to *C. neoformans* to the benefit of the host, prevent release of the capsular polysaccharide and reduce serum GXM levels. Antibodies to cryptococcal melanin (Nosanchuk et al., 1998) and cryptococcal proteins (Rodrigues et al., 2000) inhibit fungal growth, and antibodies to melanin have been shown to prolong survival (Rosas et al., 2001). Hence, AMI to *C. neoformans* is not limited to the classical antibody functions, complement activation and opsonization (Casadevall & Pirofski, 2003a, 2004). As such, AMI has great potential to enhance host immunity to *C. neoformans* by combating different pathways and mechanisms of cryptococcal virulence. Further, the proven efficacy of AMI against experimental cryptococcal infection (Casadevall & Pirofski, 2005) and the use of a mouse MAb to GXM in a clinical trial in patients with HIV-associated cryptococcosis (Larsen et al., 2005) provide a clear rationale for the development of vaccines that elicit antibodies to GXM.

**Candidate antigens for a vaccine for *C. neoformans***

**Capsular polysaccharide**

*Cryptococcus neoformans* is unique among fungi in having a polysaccharide capsule as its main virulence determinant. GXM, which is the most well-studied capsular polysaccharide determinant, is a logical vaccine antigen because it is a target of the host response and there are highly successful precedents for polysaccharide-based vaccines for encapsulated pathogens. Although purified polysaccharides can be poorly immunogenic, conjugation to a protein carrier can markedly increase their immunogenicity. The development of conjugate vaccines, which convert poorly immunogenic T-independent antigens to T-dependent antigens (Pirofski, 2001), was a landmark advance. Conjugate vaccines consisting of the capsular polysaccharides of *Str. pneumoniae* or *Haemophilus influenzae* type b (Hib) markedly enhanced the immunogenicity of the relevant polysaccharide in infants and young children. The introduction of the Hib vaccines in the mid-1980s led to the rapid eradication of Hib meningitis (Robbins et al., 1996), and use of the pneumococcal conjugate vaccine, introduced in 2000, led to a marked reduction in the incidence of invasive pneumococcal disease in children (Black et al., 2000). However, as the efficacy of pneumococcal vaccines against pneumonia is less pronounced (see Burns et al., 2005), the efficacy of polysaccharide vaccine-induced antibodies against different outcomes of infection could differ. For example, non-opsonic IgM protected against pneumococcal pneumonia through immunomodulatory effects (Burns et al., 2005), and different types of carbohydrate vaccine-induced antibodies mediated optimal protection against systemic and mucosal candidiasis (Cassone et al., 2005). Hence, a vaccine that prevents disease caused by acute *C. neoformans* infection might not work against reactivation disease, and vice versa. Of concern for a polysaccharide-based vaccine for *C. neoformans* is that polysaccharide-based vaccines are less immunogenic in individuals with HIV infection, B-cell defects, and in the elderly (Pirofski, 2001). V_{H3} immunoglobulin repertoire defects in HIV-infected individuals could translate into an impaired ability to respond to a GXM-based vaccine, as described for a pneumococcal vaccine (Abadi et al., 1998; Chang et al., 2000). However, the observation that the V_{H3} response to a pneumococcal vaccine was reconstituted in patients on HAART (Subramaniam et al., 2005) suggests such a vaccine could hold promise in the setting of immune reconstitution.

Defined antibodies to GXM can protect against experimental cryptococcosis (Casadevall & Pirofski, 2005). The efficacy of these antibodies is governed by a complex group of antibody characteristics, including isotype, idiotype and specificity (reviewed in Casadevall & Pirofski, 2005), in addition to host factors such as the genetic background (Dromer et al., 1988b) and immune status (Yuan et al., 1997; Beenhouwer et al., 2001). Since a mechanism by which these antibodies are believed to mediate protection is by enhancing the efficacy of host effector cells against *C. neoformans*, the induction of such antibodies by a vaccine could, in principle, augment host antifungal mechanisms through immunomodulation. Therefore, a vaccine that induces antibodies to GXM makes sense. However, GXM suffers from important limitations as a vaccine antigen: it is a poor immunogen and T-cell-independent type 2 antigen that does not induce affinity maturation, class switching or memory T-cells (Pirofski, 2001). Although this problem was ameliorated by conjugation to a protein carrier, (Devi et al., 1991; Casadevall et al., 1992, 2002; Devi, 1996), such a vaccine, GXM-tetanus toxoid (GXM-TT), induced non-protective and deleterious, in addition to protective, antibodies to GXM in mice (Casadevall & Pirofski, 2005). Because the nature of the GXM oligosaccharide epitopes that elicit protective antibodies have not been fully identified, various groups have focused on the identification of surrogate antigens, which would be able to induce a response to GXM epitopes that are recognized by protective antibodies.

**Peptide mimotopes**

The ability of small peptides to mimic the conformation of carbohydrate antigens (Monzavi-Karabassi et al., 2002) has led to efforts to identify peptides that mimic GXM epitopes (see Pirofski, 2001). Peptide mimetics can be selected from
libraries consisting of phage-displayed random peptides by panning the libraries with antibodies that bind to a native antigen, such as GXM. Peptides that bind the selecting antibody are referred to as mimetics; peptides that are able to elicit antibodies that bind the native antigen when they are used as immunogens are known as mimotopes. The epitopes to which protective and non-protective antibodies bind are often referred to as protective and non-protective epitopes, respectively. The goal of mimotope-based immunization is to elicit an antibody response to a protective epitope on the native antigen. Hence, the efficacy of mimotope-based vaccines depends upon the ability of antibodies to and/or that cross-react with the native antigen to prevent disease. The rationale for the development of a mimotope-based vaccine for *C. neoformans* is that, in principle, peptides that mimic defined GXM epitopes could focus the GXM response on protective epitopes, rather than on a mixture of protective and non-protective epitopes. In addition, although the specificity of carbohydrate-reactive and peptide mimotope-reactive antibodies can differ (Harris et al., 1997), the use of a peptide, rather than polysaccharide, antigen might bypass the restriction inherent in antibody responses to polysaccharides. Along these lines, peptides are T-dependent antigens, which can be highly immunogenic when conjugated to a protein carrier. Further, peptides are biochemically defined molecules that can be reliably produced in large amounts. The disadvantages of peptide-based vaccines are that there is currently no such vaccine in use in humans; focusing the response on a single epitope could enhance the risk of escape variants; and the induction of mimotope responses might be unpredictable if the desired antibodies are conformation-dependent.

GXM mimotopes selected by mouse and human antibodies to GXM have been reported. Valadon et al. (1998) selected a panel of mimetics with a high-affinity mouse IgG1 MAb that revealed unexpected complexity among GXM-induced antibodies (Nakouzi et al., 2001), but elicited a low mimotope (GXM) antibody response in mice, despite a high level of peptide-reactive antibodies. Higher-affinity peptides reactive with the same selecting MAb produced in mice a robust mimotope response focused on antibodies reactive with protective epitopes, but only after first priming with GXM-TT (Beenhouwer et al., 2002). Zhang et al. identified a decapeptide mimetic (P13) of GXM, from the same phage display library used with the mouse MAb, with a protective human IgM GXM-reactive MAb (Zhang et al., 1997). Interestingly, P13 appeared to mimic a biologically relevant epitope, because it inhibited the GXM-binding of serum antibodies from HIV-uninfected, but not HIV-infected, individuals (Zhang et al., 1997; Fleuridor et al., 1999). This finding suggested that the specificity of GXM-reactive antibodies from individuals who were at risk from cryptococcal disease was different from those who were not, and was consistent with the concept that HIV-infected individuals may have a hole in their GXM-reactive antibody repertoire (Pirofski, 2001). Immunization of mice with P13 conjugated to TT or BSA induced a mimotope response, which prolonged survival after a lethal cryptococcal challenge, as did passive administration of immune serum from these P13-conjugate-vaccinated mice to naïve mice (Fleuridor et al., 2001). Given that GXM-TT induced deleterious and non-protective antibodies (Casadevall & Pirofski, 2005), a GXM mimotope-based vaccine may hold promise for focusing the response on protective epitopes.

### Other *C. neoformans* antigens

Proteins or peptides hold promise as vaccine antigens, because they are biochemically defined and able to induce T-dependent responses. The mannoprotein component of *C. neoformans* promotes strong Th1-type immunity (Vecchiarelli, 2000). Mannoprotein immunization of mice resulted in an enhanced Th1-type cytokine response in the brain (Mansour et al., 2004). The ability of mannoprotein to enhance the host antifungal inflammatory response depends on IL-12 (Pietrella et al., 2004) and T cells (Mansour et al., 2004). A mannoprotein-based vaccine could be used to augment Th1-type immunity in individuals with weak immune responses to *C. neoformans*, with the caveat that such individuals may not be able to respond to an immunogen that requires intact T-cell immunity. The use of a mannoprotein-based vaccine in individuals with a strong immune response could run the risk of inducing a deleterious inflammatory response. Nonetheless, mannoproteins could potentially be used as adjuvants with other vaccine antigens to enhance cellular immunity to *C. neoformans*.

Other antigens that induce protective responses to *C. neoformans* include the *C. neoformans* culture filtrate antigen (CneF), a heterogeneous mixture of relatively high molecular weight polysaccharides and proteoglycans. CneF elicited delayed-type hypersensitivity and CD4+T cell activation in mice (Murphy, 1993), and CneF-immunized mice manifested prolonged survival and a lower fungal burden than controls (Murphy et al., 1998). However, CneF is a heterogeneous culture filtrate, which contains GXM and has mannoprotein as a major constituent. As such, it is not a suitable vaccine antigen. Other cryptococcal proteins that could hold promise as vaccine antigens include other mannoprotein chitin deacetylases (Biondo et al., 2002), although further preclinical development is required to assess their feasibility as antigens for humans.

### The human antibody response to GXM-based vaccines

The mouse response to GXM-based vaccines has been reviewed extensively (Casadevall et al., 2002, 2005), and the reader is referred to the foregoing articles on the topic.
Information on the human response to *Cryptococcus neoformans* vaccines is largely limited to studies that were performed in human immunoglobulin transgenic mice. However, the serum response to the investigational vaccine, GXM-TT, was examined in human volunteers. The GXM moiety in this vaccine was from a serotype-A *C. neoformans* strain (NIH 371). This study revealed that, like the response to other capsular polysaccharide vaccines, the antibody response was predominantly IgG2 (Zhong & Pirofski, 1996). Immune sera from the volunteers contained antibodies that expressed V\_H3 determinants (Fleuridor *et al.*, 1998) and promoted complement-independent phagocytosis by human polymorphonuclear neutrophils (PMNs) (Zhong & Pirofski, 1996). Two GXM-reactive IgM MAbs were produced from circulating B cells of one vaccinated volunteer. These MAbs used V\_H3 genes (Pirofski *et al.*, 1996) and one, 2E9, promoted phagocytosis and the killing of *C. neoformans* by human PMNs (Zhong & Pirofski, 1998) and prolonged the survival of mice with experimental cryptococcosis (Fleuridor *et al.*, 1998).

Human GXM-reactive MAbs were produced from human immunoglobulin transgenic mice that were vaccinated with a GXM-diphtheria toxoid (GXM-DT) conjugate (Maitta *et al.*, 2004a). These transgenic mice express human IgM, IgG2 and kappa immunoglobulin genes (Mendez *et al.*, 1997). The GXM-DT conjugate used the GXM from a serotype-D *C. neoformans* strain (ATCC24067). The MAbs produced from these mice were fully human IgMs that used the same kappa light chain. Two of the MAbs, G14 and G19, used the V\_H6 gene element and one, G15, used a V\_H3 gene element. Epitope specificity of G15 differed from that of the other MAbs and was the only MAb that protected against the *C. neoformans* challenge, although it manifested a prozone-like effect when given in high amounts. V\_H6-derived MAbs were not protective. Although they have different specificity, human V\_H3-derived and mouse GXM-reactive MAbs share a common CDR2 motif: -1----G----YY--SV-G (Maitta *et al.*, 2004a). This motif is found in six germline V\_H3 genes; V3-07, V3-11, V3-30.3, V3-30.5 and V3-33. The human GXM-reactive MAbs were germline, whereas the mouse MAbs were somatically mutated. This suggests that combinatorial diversity may be sufficient to produce antibodies to GXM in humans, which could contribute to their high level of natural resistance to cryptococcal disease. Since the GXM-reactive human MAbs used the same light-chain gene, their unique specificity could be a function of V\_H3 gene use and/or CDR3 structure. The influence of V\_H1 gene use on antibody specificity requires the study of more antibodies. Nonetheless, the difference in the GXM specificity of antibodies from HIV-infected and HIV-uninfected individuals (Zhang *et al.*, 1997; Fleuridor *et al.*, 1999) suggests that V\_H3 repertoire defects could contribute to the apparent lack of efficacy of AMI in HIV-infected individuals, despite their having high antibody levels. As immunocompetent individuals are largely resistant to cryptococcal disease while maintaining *C. neoformans* in a latent state, it is reasonable to hypothesize that naturally occurring antibodies with the characteristics of protective MAbs could contribute to resistance. As such, aberrant V\_H1 gene use, such as that observed in response to a pneumococcal vaccine (Chang *et al.*, 2000), by antibodies with different specificity could contribute to disease susceptibility in HIV-infected and other individuals with antibody repertoire defects.

### The human antibody response to peptide mimotope-based vaccines

The human antibody response to conjugates consisting of the GXM mimotope P13 and TT or DT was examined in human immunoglobulin transgenic mice expressing human IgG2 and IgG4 (Maitta *et al.*, 2004b). Vaccination with both conjugates elicited antibodies that bound to the GXM of *C. neoformans* serotype A (H99 and SB4) and D (ATCC 24067) strains (Maitta *et al.*, 2004b). This pattern of reactivity, which is similar to that described for mouse and human MAbs produced from GXM-protein conjugate-vaccinated mice, demonstrated that peptide mimotopes of GXM can induce antibodies that react with multiple GXM epitopes. However, GXM-reactive serum antibodies from P13 conjugate-immunized mice did not manifest cross-reactivity with P13, and vice versa (Maitta *et al.*, 2004b). In view of the fact that a decamer peptide can assume multiple conformations, the lack of mimetic-mimotope cross-reactivity suggests that GXM- and P13-reactive antibodies are likely to be induced by different conformations of the peptide. The availability of GXM oligosaccharides and new approaches to epitope mapping will help unravel this and other questions on the role of cross-reactivity in mimotope-induced responses to capsular polysaccharides.

P13-TT and P13-DT both induced human IgM and IgG to P13 and GXM in human immunoglobulin transgenic mice that expressed human IgG2 (Maitta *et al.*, 2004b). Conjugate-elicited antibodies expressed V\_H3 determinants that were also expressed by GXM-TT-elicited antibodies (Fleuridor *et al.*, 1998) and that were found among naturally occurring antibodies to GXM (Fleuridor *et al.*, 1999). V\_H3 gene use by GXM-reactive antibodies in the natural repertoire, as well as those induced by GXM- and P13-protein conjugates, suggests that conformational constraints, rather than the molecular form of the antigen, govern binding to certain GXM determinants. Hence, peptide surrogates might not overcome the poor response to capsular polysaccharides observed in HIV-infected and other individuals, if the response depends on components of the antibody repertoire that are depleted or disregulated. This concept...
requires further investigation, but if it is validated, it would provide a compelling reason to consider passive antibody therapy and/or approaches to antibody repertoire reconstitution or replacement in certain immunocompromised patients. One caveat regarding the immunogenicity of P13 conjugates in human immunoglobulin transgenic mice is that they were administered with the oligodeoxynucleotide CpGs, which was used as an adjuvant (Maitta et al., 2004b). CpG-treated mice manifested a GXM-reactive antibody response, including an anamnestic IgM response following a C. neoformans challenge. As CpGs enhance innate and Th1-type immunity (Ashkar & Rosenthal, 2002) and can have direct antifungal effects (Miyagi et al., 2005), their ability to enhance immunity to C. neoformans, perhaps by enhancing AMI, deserves further consideration.

The survival of P13-DT-vaccinated human immunoglobulin transgenic mice that were challenged and rechallenged with C. neoformans was prolonged compared to control mice, whereas that of P13-TT-vaccinated mice was not (Maitta et al., 2004b). Analysis of the serum antibody response revealed that P13-DT primed for a C. neoformans-induced IgG anamnestic response to GXM, whereas P13-TT primed for an IgM response. Hence, in the model under investigation, the nature of the antibody response and whether or not it was protective was a function of the protein carrier. Carrier-specific differences have been shown to influence the response to other conjugate vaccines. May et al. found that BALB/c mice immunized with a GXM peptide mimotope conjugated to glutaraldehyde-treated keyhole limpet hemocyanin (KLH) developed high levels of antibodies to GXM (May et al., 2003). However, control peptides similarly conjugated to KLH also elicited GXM-reactive antibodies that bound to a non-protective epitope, as did glutaraldehyde-treated KLH and unmodified KLH. On the other hand, the response to an experimental conjugate for another microbe was enhanced by the ability of the protein carrier to induce a microbe-specific response (Batzloff et al., 2003). These observations illustrate that current knowledge is insufficient to predict the correct conjugation partner for polysaccharide- and/or polysaccharide mimotope-based vaccines. Although the mechanisms of mimotope efficacy remain uncertain, available data show that it is not a straightforward function of the induced mimotope titer at the time of challenge. Further studies are needed to identify useful surrogates for mimotope efficacy. Nonetheless, the mimotope approach is a rationale-based approach to reverse antigen discovery, which can reveal unique protective epitopes through the use of defined antibody reagents (Pirofski, 2001).

Efforts to identify surrogates for vaccine and antibody efficacy against C. neoformans have been very difficult. Despite the knowledge gained from studies with immune sera, the functional mediator(s) of protection in such polyclonal reagents can be difficult to discern (Casadevall & Pirofski, 2004, 2005). In addition, assessments of the degree of protection afforded by polyclonal sera are hampered by its complexity and the possibility that the activity of protective antibodies, which may be present in limiting amount, can be obscured or diminished by non-protective antibodies (Casadevall & Pirofski, 2004, 2005). An advantage of using sera from human immunoglobulin transgenic mice is that, as it only contains IgM and one IgG subclass, its complexity is reduced. Maitta et al. (2004c) administered heat-treated immune sera from P13-conjugate and adjuvant (CpG)-immunized human immunoglobulin transgenic mice to BALB/c mice prior to systemic C. neoformans challenge in order to examine the efficacy of mimotope-induced human antibodies. Early sera taken from P13-DT-vaccinated mice 7 days after primary vaccination prolonged the survival of naïve BALB/c mice, whereas sera from P13-TT- and CpG-vaccinated mice did not. The functional mediator of protection in these sera was almost certainly GXM-reactive IgM, since the sera contained no specific IgG and the absorption of the sera abrogated its efficacy. In contrast, each of the late sera taken 30 days after vaccination from P13-DT-, P13-TT- and CpG-vaccinated mice prolonged the survival of naïve BALB/c mice. The functional mediator of protection in these sera was uncertain, because they each contained specific IgG and IgM. A notable aspect of these studies was that late sera from P13-TT-vaccinated mice were protective, despite the fact that P13-TT vaccination did not protect immune mice against a C. neoformans challenge (Maitta et al., 2004c). This underscored findings from studies with mouse MAbs (Casadevall & Pirofski, 2005) that, rather than being a fixed characteristic, the efficacy of AMI against C. neoformans differed as a function of the immune status of the host. Naïve hosts, albeit of a different mouse strain, were protected, but immunized hosts were not. Another interesting finding was that sera from CpG-vaccinated mice contained a specific antibody that was protective. The ability of CpGs to enhance Th1-type immunity is well documented; however, the passive transfer studies suggested they may also stimulate production of specific antibody. Although this could occur through Toll-like Receptor-9 signaling, more studies are needed to determine the mechanism by which this occurs.

**Outlook for the development of a vaccine for C. neoformans**

Available information from studies of cryptococcal pathogenesis in small animal models provides clear evidence in support of the feasibility of a vaccine for C. neoformans. The ability of defined antibodies to protect against experimental cryptococcal disease, the existence of protective antigens and the variety of mechanisms by which antibody can mediate...
protection provide a strong rationale for the development of a vaccine that induces AMI. Based on our current understanding of the virulence mechanisms of C. neoformans, including that cryptococcal disease can occur in the setting of either T-cell immunodeficiency or a reconstituted T-cell milieu, the challenge of developing a vaccine for C. neoformans will be to identify vaccine antigens that augment the host response in the setting of immunodeficiency, while dampening the response in the setting of reconstitution (and/or other states of enhanced cell-mediated immunity). Notably, the GXM and mannoproteins dampen and stimulate cell-mediated immune responses, respectively. The antibody responses to GXM among susceptible and resistant individuals are different, whereby susceptible individuals often have less GXM-reactive IgM, but more GXM-reactive IgG. The contribution of this type of antibody profile to cryptococcal pathogenesis has not been established. However, high antibody amounts can enhance experimental cryptococcosis, certain IgMs are effective against experimental cryptococcosis and innate immunity to encapsulated pathogens can depend on IgM (Brown et al., 2002). Hence, it is logical to hypothesize that a vaccine for C. neoformans should induce a balanced immune response and promote cooperative interactions between innate and cell-mediated immunity. The variety of mechanisms of antibody action against C. neoformans (Casadevall & Pirofski, 2003a, 2004, 2005) suggests it should be possible to induce immunostimulatory and immunoregulatory responses (Fig. 2). As such, the challenges that lie ahead are to identify targets that induce such antibodies, and to determine whether or not one or more than one vaccine will be needed to protect against acute and reactivation disease, and protect individuals with different immune status.

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**References**


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