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Antibody-mediated protection through cross-reactivity introduces a fungal heresy into immunological dogma

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It is hard to believe that only a decade or so ago the question of whether specific antibodies protected against fungal pathogens was considered controversial (reviewed in (5)). Since the landmark paper by Dromer et al in 1987 showing that a monoclonal antibody (mAb) to *C. neoformans* polysaccharide was protective against experimental cryptococcal infection in mice (10), dozens of studies have established conclusively that certain antibodies are protective against fungi. Protective mAbs have now been described for *C. albicans* (13,16,24,42), *C. neoformans* (10,11,21,26,38), *A. fumigatus* (7), *Pneumocystis* spp (12), and *H. capsulatum* (28). In recent years two antibodies have entered clinical evaluation for fungal diseases (20,29). A consensus has now emerged that the inability of immune sera to mediate protection against fungi reflected inadequate amounts of protective antibody and/or the simultaneous presence of protective and non-protective antibodies, rather than a fundamental inability of antibody to protect against fungal pathogens. In support of this concept, in addition to protective mAbs, non-protective mAbs to *C. albicans*, *C. neoformans*, and *H. capsulatum* have been described (13,25,28). Nonetheless, much remains to be learned about the nature of protective antibodies to and the relationship between the natural antibody response, resistance and susceptibility to fungal pathogens, since hypogammaglobulinemia is not generally associated with the development of fungal disease and antibody responses to certain fungi and fungal targets can be a marker of disease, rather than immunity (19,30).

Perhaps the central event in this odyssey was the application of hybridoma technology to studies of antibody immunity to fungi. This approach made it possible to characterize the functional efficacy of individual immunoglobulin molecules. Hence, studies in medical mycology revealed a new immunological paradigm in which the protective potential of immune sera is a function of the aggregate activities of immunoglobulin molecules instead of a singular

property. This view challenged the traditionally held dichotomy in which cellular immunity is responsible for resistance to intracellular pathogens and antibody immunity is responsible for resistance to extracellular pathogens. In addition, in recent years studies with fungi also threaten to tear down another pillar of immunological dogma, namely, that protective immune responses are pathogen-specific.

A multitude of targets for antibody mediated immunity (AMI)

The current approach of making mAbs to fungal surfaces and then evaluating their efficacy in animal models has revealed numerous antigens that can elicit protective antibody responses (Table 1). Protective mAbs have been made against classical fungal surface antigens such as mannans, glucans and glucuronoxylomannans. Most interestingly, immunization with fungi and fungal lysates has produced unexpected results, identifying antigens that were hitherto not suspected to be targets of AMI, including surface heat shock (23) and histone-like proteins (28). There is now evidence that proteins, polysaccharides, pigments and even glycolipids are also targets for protective antibody responses (Table 1).

In this issue yet another cryptococcal target is described in the form of beta-glucan (34). The addition of beta-glucan to the list of *C. neoformans* targets for protective antibodies is important for biological and practical reasons. Of fundamental biological interest is that beta glucans appear to be emerging as potentially 'universal' targets for antibody immunity on fungi. Of practical importance, antibodies to beta glucans have now been shown to protect against *Aspergillus spp*., *C. albicans* and *C. neoformans*.

Common targets can induce protective antibodies to unrelated fungal species

The discovery of so many fungal targets for antibody immunity fungi is surprising in view of how long the view that antibody immunity did not contribute to host defense against

fungi prevailed. In fact, the ever expanding number of potential targets on certain fungi, like *C. neoformans*, is paradoxical when compared to the limited number of targets for antibody immunity that have been identified on microbes for which the primacy of antibody immunity has been established and accepted. Hence, the number of different targets that are identified for antibody immunity to different pathogens appears to reflect a combination of the approach to antigen discovery, available knowledge on the virulence of and prevailing beliefs about host defense against the relevant microbe. Collectively, the use of mAbs to study antibody immunity to fungi provided a proof of principle that fungal antigens were viable targets for antibody immunity. Paradoxically, the fact that such a proof of principle was not required for viral or bacterial diseases may have held back the discovery of novel targets for viruses and bacteria.

Interestingly, the concept that 'common' or 'universal' targets could induce protective antibodies to different fungal species was readily embraced by the field and pursued experimentally. This is surprising in view of the fact that some fungal targets are conserved among phylogenetically distant fungi belonging to different species. To put the phylogenetic differences into perspective, *C. albicans* and *C. neoformans* belong to the ascomyces and basidiomyces groups, respectively, which may have diverged over 1 billion years ago. An early example that universal fungal targets can induce protective antibodies was the demonstration that mAbs mimicking killer toxin were fungicidal to *C. albicans* and *Aspergillus* spp (33,39). The efficacy of the mAbs was attributed to the expression of killer toxin by diverse fungal species. Similarly, antibodies to melanin inhibited the growth of both *C. neoformans* and *Fonsecaea pedrosi* (Table 1). Another dramatic example of the efficacy of a universal target is that a conjugate consisting of the poorly immunogenic antigen laminarin, which is composed of beta-glucans, and diphtheria toxoid elicited antibodies that protected against both *C. albicans* and *A.*

fumigatus, two ascomycetous fungi (42). A mAb to laminarin beta glucan directly inhibited the growth of *C. albicans in vitro*, suggesting that the protective effect of immune sera to beta-glucan involved the production of antibodies with direct antifungal activities (42). Since beta-glucans are found in the fungal cell wall this inhibitory effect could reflect antibody-mediated interference with cell wall remodeling during replication. A similar mechanism may account for the antifungal effect of melanin-binding antibodies. Rachini et al have now shown that the same mAb that protected against *C. albicans* and *A. fumigatus* (mAb 2G8) is also active against *C. neoformans* (34). Therefore, beta-glucans are also targets of antibody immunity in a basidiomycetous fungus, even though the basidiomyces and ascomycetes are different types of fungi with very different cell wall organization. The ability of mAb 2G8 to bind to and inhibit growth of both types of fungi establishes that fungal antigens that are common to different species are viable targets for antibody immunity.

The cell wall as an Achilles heel.

The fungal cell wall is a remarkably complex structure that remains poorly understood with regards to its architecture and antigenic composition, yet it is a major target for the immune system (27). Most prior studies of antibody immunity to fungi have focused on non-cell wall fungal species-specific antigens such as the GXM of *C. neoformans* and the aspartyl proteases of *C. albicans*. However, evidence that antibodies to beta-glucans, Hsp90, histone-like proteins and melanin can each inhibit fungal replication suggests that cell wall antigens provide a vulnerable Achilles heel for antibody-mediated protection against fungi. The cell wall is highly immunogenic and antibodies to cell wall determinants, including beta glucans, are produced during fungal infection. However, prior studies of passive protection with immune sera elicited by fungi produced inconsistent results. This can be explained by inadequate amounts of

protective antibodies or that sera contain both protective and non-protective antibodies. In addition, direct antifungal effects may require a higher concentration of cell-wall specific antibodies than are produced in the course of the immune responses elicited by infection.

Mechanisms of antibody mediated protection

Classical mechanisms of antibody action include direct effects, such as toxin and viral neutralization and cooperative effects, primarily mediated through effector cells, such as enhancement of phagocytosis by opsonization, complement activation and fixation and antibody dependent cellular cytotoxicity (ADCC) (6). In recent years additional mechanisms of antibody action against fungi have been revealed, including growth inhibition (42), inhibition of biofilm formation (22), inhibition of adherence (24), inhibition of germination (24) and direct antifungal effects (24). For antibodies to *C. albicans* mannoproteins, *P. carinii* surface antigen and *C. neoformans* GXM, the Fc region and/or complement were essential for antibody efficacy (15,41,44), whereas the activity of antibodies to other *C. albicans* mannoproteins (MP65) and HSP 90 (9,29) is mediated by antibody fragments (Fabs) and does not require Fc regions. Notably, antibodies to *C. albicans* MP65 and SAP-2 that lack Fc regions were shown to inhibit fungal adherence to epithelial cells (9). The efficacy of antibody fragments against experimental candidiasis in mice suggests they may hold promise for avoiding the formation of potentially detrimental immune complexes and wanted antibody responses to Fc regions that can be induced when the antibody is derived from a different species. However, since Fc regions are necessary for the efficacy of antibodies to other fungal targets, a greater understanding of mechanisms of antibody efficacy against different fungal targets is needed to develop rationally based therapeutic antibody agents for fungi. While there is currently insufficient evidence to suggest that antibodies to fungal targets are more effective against systemic or mucosal disease, or both,

it is possible that antibodies that mediate protection by blocking adherence might be more effective against mucosal disease, whereas those that mediate protection by enhancement of effector cell phagocytosis might be more effective against systemic disease.

A changing immunological landscape: the emergence of cross reactive targets for fungi.

The demonstration that many antibodies to fungal antigens can protect against fungal diseases has already overturned the old notion that host defense against mycoses is solely the purview of cell-mediated immunity. The demonstration that antibodies to beta-glucan have biological activity that translates into a host benefit issues yet another challenge to the longstanding immunological dogma that antibodies to common or universal antigens are not protective. Often, such non-pathogen-specific antigens are referred to as 'cross-reactive'. While such determinants, of which beta glucans are an example, can be viewed as 'cross-reactive' by virtue of being present across different fungal species, functionally, they are 'common' or 'universal' components of fungal cell walls. Whether they are viewed as 'cross-reactive' or as 'common' or 'universal' the concept that such determinants are viable targets for antibody immunity represents a major shift from classically held paradigms of immunological thought.

With the exception of certain viral vaccines that use the entire microbe as a target, available vaccines consist of components, or subunits, of microbes that cause viral, bacterial or toxin-mediated diseases. Many of these diseases are unique in having a single, pathogen-specific determinant, such as a capsular polysaccharide or a toxin that is responsible for virulence and is the target for the protective antibody response. The existence of singular determinants of virulence amongst the first microbes for which successful vaccines were developed led to the prevailing dogma that non-pathogen-specific, or cross-reactive, determinants do not induce protective responses. As such, there has been little or no appreciation of the potential for

'common' cross-reactive antigens to confer immunity against bacteria and viruses. Nonetheless, protection against more than one pathogen with a single vaccine or antiserum could provide the host with an economical mechanism to protect against several pathogens at once without having to experience the dangers associated with encountering each pathogen. Fungi are ripe to inform us on this potential as the remarkable finding that mAb 2G8 (to laminarin) protects against *C. neoformans*, *C. albicans* and *Aspergillus* demonstrates (34,42). Attempts to harness immunomodulators as antimicrobial agents underscore the fact that infectious diseases are a manifestation of host damage stemming from host-microbe interaction and that many infectious diseases result in similar immunopathology and damage. For example, fungal beta glucans are highly inflammatory (45). Therefore, although antibodies to beta glucans inhibit fungal growth *in vitro*, their effect *in vivo* may be as immunomodulators, because their activity prevents the development of inflammation.

While the importance of acquired, or pathogen-specific, antibody immunity for host defense against infectious diseases has been long recognized, the importance of naturally occurring, or non-specific antibody immunity has recently emerged as a critical aspect of host defense against a variety of pathogens. Naturally, occurring IgM antibodies are part of the pre-immune repertoire. They are derived from a self-renewing population of B-1 cells that arises without antigenic stimulation, but responds to cytokines and common, or universal, pathogen associated determinants, including carbohydrates. Naturally occurring IgM antibodies have been shown to be essential for the resistance of naïve hosts to experimental infections with a diverse array of bacteria, viruses and parasites (1,3,4,18,35). The mechanism by which these antibodies mediate protection remains a subject of active investigation. The importance of the naturally occurring antibody repertoire for resistance to fungi has yet to be rigorously explored. However,

evidence that antibodies to *C. albicans* and *C. neoformans* are ubiquitous in human sera suggests that human resistance to disease with these fungi could in part be attributable to naturally occurring carbohydrate-reactive antibodies.

The promise for antibody-based therapeutics and vaccines for fungal diseases.

The finding that it is possible to protect against multiple fungal pathogens by eliciting an antibody response to a single antigen introduces the promise of broad-spectrum vaccines and therapeutic antibodies. The findings of Rachini showing that beta-glucan antibodies protect against *C. neoformans* (34), combined with the earlier report that such antibodies also protect against *C. albicans* and *A. fumigatus* (42), suggest it may be possible to protect against three major fungal pathogens with a single vaccine. Furthermore, it may be possible to develop antibodies to beta-glucan for passive therapy of the diseases caused by each of these fungal pathogens. Since beta-glucans are common in fungal cell walls it is likely that antibody responses to this polysaccharide may also be protective against other fungal pathogens. Hence, these findings herald the promise of vaccines targeting common fungal antigens. Since *C. albicans*, *A. fumigatus*, and *C. neoformans* are common pathogens of individuals with impaired immunity it may be possible to simultaneously protect against these microbes by vaccinating populations at risk. Although developing vaccines for immunocompromised individuals poses special challenges, it is noteworthy that this goal has been accomplished, most notably the prevention of varicella in children with hematologic malignancies. In addition, the use of adjuvants and immunomodulators holds promise for enhancing the immunogenicity of vaccines in immunocompromised patients.

The discovery of numerous antigens on the fungal cell wall that elicit protective antibody responses raises the possibility of obtaining synergistic effects by designing vaccines with

multiple antigens and/or passive therapies that combine antibodies with different specificities. Given that some of the antigens described are polysaccharides and proteins, it is attractive to consider the possibility of designing protein-conjugate vaccines that elicit protective antibodies to both types of moieties.

The prospects for vaccines for fungi: A lesson from studies of antibody immunity

In contrast to viral and bacterial diseases, mycology is a latecomer in teaching us about host defense and establishing immunological paradigms. This undoubtedly reflects the fact that until relatively recently very little work was done to investigate the pathogenesis of and immunological responses to fungal pathogens, possibly because these microbes did not emerge as major clinical problems until the second half of the 20th century. Since most of the historical concepts of host defense upon which vaccine development and serum therapy were based were established by detailed studies of bacteria and viruses, it is not surprising that the concept of inducing protection by targeting a common or universal antigen might appear heretical according to immunological dogma. However, given the enormous antigenic and phylogenetic differences between fungal and bacterial pathogens it is likely that immunological studies of medically relevant fungi will establish new principles in host defense that were not apparent from studies with prokaryotes or viruses. Hence, today's heresy may be tomorrow's useful dogma.

Table 1. Fungal antigens shown to elicit protective antibody responses.

Fungus	Antigen	Reference
Aspergillus spp	Beta-glucans	(42)
	100 KDalton cell wall protein	(7)
	Allergen Asp f 3	(17)
Candida albicans	Mannoproteins, secretory aspartyl proteinase (SAP)-2	(9,13,14,40)
	Aspartyl protease	(8)
	Killer toxin receptor	(32,33)
	Heat shock protein 90	(23)
	Beta-glucans	(42)
Cryptococcus neoformans	Glucuronoxylomannan	(10,11,26,31,38)
	Melanin	(37)
	glucosylceramide	(36)
	Beta-glucans	(34)
Fonsecaea pedrosi	melanin	(2)
Histoplasma capsulatum	Histone-shock like protein	(28)
Pneumocystis spp.	surface glycoproteins, Kex1	(12,43)

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