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Vaccines for invasive fungal infections

Brad Spellberg

Address: Division of General Internal Medicine, Los Angeles Biomedical Research Institute, Harbor–University of California Los Angeles (UCLA) Medical Center, 1124 West Carson Street, Liu Vaccine Center, Torrance, CA 90502, USA

Email: bspellberg@labiomed.org

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Abstract

Morbidity and mortality from invasive fungal infections remain unacceptably high despite availability of new antifungal agents, underscoring the need for more effective preventative strategies. Due to our enhanced understanding of the host defense and pathogenetic mechanisms that lead to invasive fungal infections, it should be feasible to develop vaccines targeting these infections. A common immunological theme across many vaccine candidates for invasive fungal infections has been the need to activate a cell-based, pro-inflammatory, Th1 or Th17 immune response to improve phagocytic killing of the fungi. Since neutralization of virulence factor functions has not been required for many active vaccines to function, the antigenic repertoire available for testing should not be limited to virulence factors. With expansion of our fundamental understanding of the immunology of fungal infections, the biggest barrier to development of fungal vaccines is the lack of available capital to translate discoveries made at the bench into biological agents used at the bedside. Continued education on the importance and feasibility of vaccination for such infections, combined with continued development of vaccine antigens and adjuvants, is necessary.

Introduction

Invasive fungal diseases often take hold when a person's natural defenses are weakened. These infections frequently occur in hospital settings. For example, candidemia, an infection caused by one of several species of the yeast *Candida*, typically occurs after a patient's normal bacterial flora are wiped out by antibiotics, or their skin and gut mucosa are breached by central venous catheters or surgery. Candidemia is now the fourth most common bloodstream infection in hospitalized patients both in the United States and many European countries. And the death rate from such *Candida* infections remains about 30–40%, even after treatment with antifungal therapy. Given their increasing frequency and unacceptably high morbidity and mortality rates, prevention of invasive fungal infections has become of paramount importance.

Vaccination of high-risk groups is a particularly promising strategy to prevent invasive fungal infections because easily identifiable risk factors are clearly defined for many such infections [1]. Furthermore, development of these risk factors precedes infection, affording a window of opportunity to vaccinate acutely at-risk patients before the onset of infection.

For years the development of fungal vaccines has lagged behind that of vaccines formulated to attack viruses and bacteria. One barrier has been the widespread belief that most patients who develop life-threatening fungal infections have profound defects in immunity—for example, those whose immune systems have been impaired by cancer chemotherapy. One major concern about vaccinating such patients against invasive fungal infections has been the assumption that the immune systems of patients at risk for these infections are too weak to respond vigorously to vaccination.

However, only some 10–20% of patients who develop bloodstream infection from *Candida* are seriously immunocompromised. The large majority of patients develop the infection because of breaches in anatomical defenses and altered normal flora in hospitalized patients. For

example, patients become susceptible to this infection in the hospital because of exposure to broad spectrum antibiotics, central venous catheters (which breach skin defenses), surgery (which breaches gut mucosa defenses), and prolonged periods without feeding, which can lead to gut mucosal wasting (from lack of use). Such patients have relatively intact leukocyte function and should respond to vaccination. In addition, there is extensive literature confirming the immunogenicity and efficacy of vaccines even in patients with extremely weakened immune systems—for example, those with active leukemia, HIV infections, or receiving immune-suppressing corticosteroids [5-13]. Because of such data, it is recommended to administer vaccines (aside from live attenuated vaccines) to patients with cancer and HIV despite their immunocompromised conditions [14,15]. Since patients with profound lymphocyte defects can respond to vaccination, it is likely that acutely ill hospitalized patients whose primary host defense defect is anatomical will also respond to vaccination.

In recent years, a number of research groups around the world have begun to focus on creating vaccines against some of the most serious and deadly fungal infections. We are closer than ever to bringing a protective vaccine to the clinic, but a number of technical and economic barriers remain to be overcome before the first such vaccine is available for use in humans. The purpose of this review is to summarize the current status of candidate vaccines, and discuss barriers and solutions to their continued development.

Candida vaccines

By far the most common causes of invasive fungal infections are members of the genus *Candida* [2]. Population-based surveys in the United States have reported that the annual incidence of disseminated candidiasis is approximately 20–24 cases per 100,000 population (60,000–70,000 cases per year in the United States) [1]. As each of these cases adds tens of thousands of dollars to hospitalization costs, it has been estimated that the healthcare cost associated with hematogenously disseminated candidiasis is $2–4 billion/year in the United States alone [2]. Hence, a vaccine that could prevent or ameliorate these infections would be of major benefit to US and indeed global health, and would be of significant value to national healthcare systems.

Animal models confirm that disruption of normally protective, anatomical barriers is fundamental to the pathogenesis of disseminated candidiasis, irrespective of underlying immune function [3,4]. Thus, the predominant risk factors for disseminated candidiasis are common iatrogenic and/or nosocomial conditions that disrupt protective anatomical barriers or result in a substantial increase in the colonization burden of *Candida spp.*, such as indwelling plastic catheters, abdominal or cardiac surgery, prolonged hospital stay, stay in an intensive care unit, and receipt of broad-spectrum antibiotics. Patients with such risk factors should have preserved adaptive immunity, and would be expected to mount immune responses to vaccination.

Recently, vaccines targeting *Candida* have been described in preclinical settings. For example, a protein conjugate vaccine consisting of laminaran (algal glucan) linked to diphtheria toxoid as a carrier protein resulted in significant protection against disseminated candidiasis and vaginal candidiasis in murine models [16,17]. The vaccine was cross-protective against *Aspergillus* as well, suggesting it may be effective against all fungi that contain glucan in their cell walls. Most recently, the investigators established the efficacy in mice of related vaccines with an oil-in-water adjuvant (MF59), which has been used safely in experimental vaccines in humans [18], indicating that the laminaran vaccine is a promising candidate for translation to clinical trials.

A similar vaccine using candidal mannan conjugated to human serum albumin as a carrier protein was found to be immunogenic in rabbits, although efficacy in the face of infectious challenge was not studied [19]. Other anticandidal vaccines that have been assessed in published preclinical studies have focused on immunizing with candidal heat shock proteins [20], conjugates of peptides with fungal mannans [21], other candidal surface proteins (e.g., HYR1), which resulted in impressive protection in a systemic infection model [22], and use of heat-killed *Saccharomyces cerevisiae* as a panfungal immunomodulatory agent based on carbohydrate cross-reactivity [23]. Such studies continue to build upon the fundamental knowledge of protective immunity against *Candida*, which should ultimately lead to development of a clinically useful vaccine.

The candidal vaccine furthest along the developmental pathway is based on the agglutinin-like sequence (Als) family of proteins from *Candida albicans*. Vaccination with the recombinant N-termini of the candidal surface adhesins Als1p or Als3p (rAls1p-N or rAls3p-N) protected mice from otherwise lethal disseminated candidiasis, and also reduced fungal burden in a vaginitis model and a steroid-treated oropharyngeal candidiasis model [24-30].

A detailed mechanistic understanding of Als vaccine protection is now available, which elucidates fundamental requirements of host defense against disseminated candidiasis. Effective vaccination with rAls3p-N,

protecting against lethal systemic infection, required intact proinflammatory, type 1 immune responses (i.e., interferon-gamma [IFN-γ]), and also a more recently described T cell subset, Th17 cells [30]. Th17 cells mediate phagocyte recruitment to and activation of phagocytes at sites of infection. Vaccine efficacy did not require Th2 responses (i.e., interleukin [IL]-4), and was independent of antibody or B cells [24,26,29-30]. These results elucidate several critical features of vaccinations against extracellular fungal pathogens. Firstly there is likely a requirement for enhancement of phagocytic host defense mechanisms by vaccine-responsive T cells or by protective antibody. Secondly, it is not necessary to neutralize virulence factor functions in order to achieve protection with a vaccine—use of the vaccine to target the organism for superior killing by the innate immune system can result in protection even without blocking virulence factor functions. These findings open the door to identification of a variety of antigens as vaccine candidates even though those antigens are not "virulence factors" for fungal pathogens.

Establishment of Als efficacy in combination with an aluminum-based adjuvant is a critical milestone, because aluminum derivatives have been widely used in US Food and Drug Administration (FDA)-approved vaccines. Hence, a dosing schedule, route of administration, and adjuvant have now been identified for rAls3p-N that supported an "Investigational New Drug" (IND) application to enable clinical testing to begin, after Good Manufacturing Practice (GMP)-compliant manufacturing was achieved. Indeed, a Phase I clinical trial (NCT01273922) has been completed with the vaccine, with results pending.

The costs of preparation for an IND application supporting Phase I trials for such vaccines are significant. It takes several million dollars just to develop GMP-compliant manufacturing, as well as additional costs for preclinical toxicity studies using GMP-compliant material, and also the costs of IND filing, clinical protocol development, and the costs of executing the study. These costs represent a major barrier to development of vaccines for invasive fungal infections in general, and are an even greater barrier for infections caused by fungi other than *Candida*, which have smaller perceived markets to attract investment capital.

Aspergillus vaccines

Aspergillus is the second most common cause of nosocomial, invasive fungal infections, with an incidence of approximately 5 per 100,000 population in the United States [2]. Both the potential advantages of, and barriers to, a vaccine against aspergillosis are greater than with invasive candidiasis. For example, aspergillosis infection has an extremely high mortality rate despite antifungal therapy (45% to >80% [1]). Conversely, a particular barrier for development of a vaccine is that virtually all patients with invasive aspergillosis are highly immunocompromised, which could make vaccination more problematic.

As, generally, only severely immunocompromised patients develop aspergillosis, it is not certain that vaccination will prevent aspergillosis in such patients. However, as mentioned above, there is ample evidence that even patients with profound immune dysfunction can mount responses to vaccines. As with *Candida*, the risk factors for aspergillosis are well understood, and the infection tends to occur after multiple weeks at risk. Hence, it should be feasible to vaccinate a focused target population prior to onset of infection.

Recently, the feasibility of vaccination of mice with crude antigen preparations from an *Aspergillus* strain, *A. fumigatus*, has been demonstrated, even in animals that were subsequently immunocompromised [31,32]. In these studies, vaccination was found to improve survival against both inhaled and intravenously administered fungi. Furthermore, similar to the rAls3p-N vaccine against disseminated candidiasis, the efficacy of *A. fumigatus* crude protein vaccination required the presence of IFN-γ and was mediated by CD4+ lymphocytes in adoptive transfer studies. However, a crude protein preparation is not going to support a clinical development program. A defined antigen preparation that can be manufactured to GMP-compliance must be identified. Nevertheless, these studies continue to support the idea that vaccination against invasive aspergillosis may be a practical proposition.

Building upon these earlier studies, more recent work has confirmed that recombinant protein antigens from *Aspergillus* can be used to induce type 1, cell-mediated immune responses that protect mice against invasive aspergillosis. Specifically, intranasal administration of the recombinant allergen, Asp 16 f, in conjunction with CpG oligonucleotides as a type 1-promoting adjuvant, was shown to improve the survival of cyclophosphamide-treated mice subsequently infected with inhaled *A. fumigatus* [33]. *Ex vivo* dendritic cells pulsed with *Aspergillus*, or with fungal RNA, induced Th1 cell polarization and improved survival of allogeneic stem cell-transplanted mice infected with *A. fumigatus* [34]. (The function of the stem cells was to mimic the allotransplant population undergoing treatment for cancer, which is the highest-risk patient population.) Similarly, mouse dendritic cells transfected with an IL-12-expressing adenoviral vector and exposed to *A. fumigatus* stimulated type 1

immune responses *in vivo* after adoptive transfer, which was protective during a subsequent lethal challenge. The dendritic cells were transfected with IL-12-expressing adenovirus to force them to produce IL-12, which activates IFN-g/Th1 responses.

Such novel techniques may lead to future breakthroughs in immunoprophylaxis against aspergillosis, although much work remains to establish GMP-compliant manufacturing, safety, and clinical development strategies for such technology. In the meantime, continued study to identify relevant proteins from *Aspergillus* with which to immunize, and to identify a dosing regimen and adjuvant that can be manufactured to GMP-compliance and tested in clinical trials, is warranted.

Along these lines, Ito et al. [35] have recently determined that the immunodominant antigen in their previously published *Aspergillus* crude extract was Asp f 3. Subcutaneous vaccination of mice with recombinant Asp f 3, or specific fractions thereof, protected mice from subsequent lethal inhalational challenge with *A. fumigatus*. While protection with the soluble form of protein required the use of TiterMax adjuvant (which is too toxic for use in humans), the investigators also demonstrated that a protein precipitate form of the vaccine, administered as a suspension in methylcellulose carrier, was also protective, and so may be usable in humans. With this protein precipitate vaccine, it may be feasible to achieve GMP-compliant manufacturing and continued development and optimization as a lead vaccine candidate against invasive pulmonary aspergillosis.

Cryptococcus vaccines

Cryptococcus causes life-threatening infections in patients with substantially compromised T cell-mediated immunity, whether congenital, corticosteroid-induced, or due to acquired immune deficiency (e.g., HIV infection). A population-based estimate of 3 cases of invasive cryptococcal disease per 100,000 population in the United States places this disease third, behind *Candida* and *Aspergillus*, as an organism-specific cause of invasive fungal infections [2]. As with aspergillosis, a vaccine against cryptococcal infection must be effective in patients with substantial T-cell immune deficiency. The rationale for selecting antigens that could comprise an active vaccine against *Cryptococcus* has been summarized in an excellent, recent review published by one of the leading groups in the field [36]. One antigen identified in this study, the glucuronoxylomannann (GXM) capsule of *Cryptococcus*, has long been known to be a predominant cryptococcal virulence factor that functions by suppressing the host inflammatory response and preventing opsonophagocytosis of the fungus [37]. Antibodies specific to the capsule are capable of overcoming the capsule's protective effect and enhancing opsonophagocytic killing of the fungus by host phagocytes [38,39]. However, natural infection may induce nonprotective antibodies [40]. Several antigens have been found to induce protective immunity against cryptococcal infection in mice. The previously mentioned protein-conjugated laminaran vaccine cross-reacts with *Cryptococcus*, inducing specific antibodies that induced opsonophagocytosis and reduced fungal burden in healthy mice and even in neutropenic mice (although not studied in T-cell deficient or steroid-treated mice, which would better reflect the target population in humans) [41]. Other examples of antigens that have been found to have efficacy in mice against cryptococcal infection include peptide mimotopes of the GXM capsule [36,42] and complex mixtures of proteins resulting from biochemical extraction of cell surface mannoproteins or culture filtrate antigens (CneF) [36].

A critically important lesson in fungal immunization has been learned as a result of the extensive antecedent research into vaccine immunity for cryptococcal infections, which clearly established the role of mannosylation in inducing protection of protein vaccines against *Cryptococcus*. In elegant work conducted by Specht et al. [43], the same recombinant protein produced separately by *Pichia pastoris* and *Escherichia coli* (which formed mannosylated and unmannosylated versions of the protein, respectively) induced disparate immune responses and protection. Induction of potent T cell-mediated immune responses, and protection against infection, required the presence of mannosylation and hence production of the protein in the yeast.

These results are concordant with similarly conducted experiments using proteins from model systems (e.g., ovalbumin with transgenic T cells specific to ovalbumin). For example, Lam et al. [44] produced recombinant ovalbumin that either was or was not mannosylated during production. Yeast-mannosylated ovalbumin induced significantly greater T cell proliferation than unmannosylated ovalbumin produced in *E. coli*. Furthermore, the stimulatory effect was attributable to O-mannosylation rather than N-mannosylation. Chemical deglycosylation abrogated the stimulatory effect of the mannosylated protein, as did competitive inhibition with free *S. cerevisiae* mannans not attached to protein during lymphocyte co-culture, confirming that the mannosylation of the protein antigen was responsible for the immunological effect. The same group found a similar immunostimulatory effect of mannosylated antigens on CD8+ T cell function [45]. Mannosylation significantly enhanced CD8+ T cell proliferation, and also secretion of proinflammatory cytokines, such as tumor necrosis factor (TNF) and IL-12.

Collectively, these results are of fundamental importance to recombinant protein vaccines targeting fungal pathogens in general, and suggest that large-scale production of such protein vaccines should use yeast to induce mannosylation, or if *E. coli* is used, that chemical conjugation to fungal mannans may boost immunity and protection.

Endemic mycoses

Unlike other invasive fungal infections, the endemic mycoses routinely infect immune-normal individuals. Consequently, vaccination for endemic mycoses is not plagued by concerns about the immune responsivity of compromised host populations. However, the endemic mycoses also present two specific barriers to vaccination that are more problematic than for other invasive fungal infections. First, vaccination would target the general population living in endemic areas and would require prolonged—or lifelong—immunity to be maintained. In contrast, vaccination against other invasive fungal infections targets a carefully selected population of acutely at-risk patients and protection is typically only required for short periods of time (i.e., weeks). The risk–benefit ratio of large-scale vaccination programs targeting the general population with a lower overall attack rate is quite different from vaccination programs that would target patients who are acutely at particularly high risk for infection. Furthermore, maintenance of immunity would undoubtedly require booster immunizations over time, further affecting the risk–benefit ratio of the program.

A second barrier for vaccines against endemic mycoses is that, although the number of invasive infections per year caused by the endemic mycoses is quite large, these infections occur only in limited geographical areas, and the number of patients who develop significant sequelae from infection represents a small fraction of the total infected population. The combination of geographical restriction and relatively rare serious sequelae have left the unfortunate impression among those in charge of government or private capital that vaccines targeting the endemic mycoses are not worthy of investment. It is this misperception of the economic realities governing a vaccine for endemic mycoses that has been the biggest barrier to development of vaccines for these diseases, by far.

Despite the unfortunate lack of enthusiasm among those in control of investment, the quantity and quality of science generated by dedicated investigators seeking to develop vaccines against endemic mycoses is impressive. For example, the *Blastomyces* adhesin 1 (BAD1) antigen (formerly known as WI-1) from *Blastomyces dermatitidis* has been identified as a crucial virulence factor for the fungus, and the immunodominant focus of the mammalian immune response to infection [46]. Vaccination with purified BAD1 protected mice from otherwise lethal infection caused by *B. dermatiditis* [46]. As with *Candida* and *Aspergillus* vaccines described above, CD4+ T lymphocytes, and proinflammatory cytokines such as TNF and IFN-γ were required for host protection [47-49]. Immunocompromised mice were able to overcome deficits in CD4+ cell function by using memory CD8+ cells as sources for proinflammatory cytokines [48,49].

Work on a vaccine targeting another endemic mycosis, coccidioidomycosis, has been ongoing for more than a century [50]. Most recently, efforts have focused on multivalent vaccines, comprised of complexes of protein antigens administered with combinations of adjuvants [51,52]. One such combination vaccine has even been administered in a nonhuman primate model, and a dose–response effect was demonstrated [53]. The antigen in this vaccine was a recombinant protein chimera in which the first 106 amino acids of the antigen 2/proline-rich antigen (Ag2/PRA) cell wall antigen from *Coccidioides* were combined with the *Coccidioides*-specific antigen (CSA). The adjuvant used was a combination of CpG oligonucleotides with Montanide oil adjuvant. Use of a recombinant protein is clearly advantageous from a manufacturing perspective, and both CpG oligonucleotides and Montanide adjuvants have been used in clinical trials previously. These factors, combined with the general lack of toxicity seen in the primate study, underscore the potential to move forward into clinical trials with this or a similar vaccine candidate.

Nevertheless, as mentioned, significant perceived economic barriers remain to developing a *Coccidioides* vaccine, just as for a *Blastomyces* vaccine. These perceived economic barriers have prevented access to the capital required to enable implementation of full-scale GMP-compliant manufacturing and subsequent clinical protocol development. It is particularly unfortunate that these economic barriers are delaying implementation of a development plan for these vaccines, because extensive evaluations have confirmed the large economic burden and obvious societal cost–benefit of a vaccine for both *Coccidioides* and *Blastomyces* [54-56]. It is hoped that continued education about the economic burden of the endemic mycoses, in both human and veterinary populations, and continued study of adjuvants to maximize efficacy will ultimately enable clinical development of vaccines for these diseases.

Conclusions

With the aging global and US populations, increasingly intensive medical treatments of critical illnesses, and increasingly aggressive immune-suppressive treatment of patients with cancer, the incidence of invasive fungal

infections will continue to rise over the coming decades. Mortality rates associated with these diseases remain high despite the availability of new antifungal agents. Together, these factors make prevention of these infections by vaccination highly desirable. Due to several major advances in understanding of host defense and pathogenetic mechanisms that underlie invasive fungal infections, we are now in a position to begin developing such vaccines. For most active vaccines studied to date against invasive fungal infections, the key to protection has been induction of cell-mediated, pro-inflammatory, Th1 or Th17 responses, which improve phagocytic killing of the fungus. It is also clear that antigens targeted for vaccination need not be restricted to virulence factors, which markedly increases the antigen repertoire available for testing. Also, the concept of niche vaccination of acutely at-risk patients or patients in restricted geographical areas is a new idea that opens doors in vaccinology.

The lack of complete understanding of the market potential for such vaccines has created significant impediments to the availability of the requisite capital to develop these vaccines. Continued education about the economic importance of vaccines for invasive fungal infections, combined with continued development of well-defined antigens and effective adjuvants with a track record of safety, should enable such vaccines to enter clinical testing in the coming decade.

Abbreviations

Als, agglutinin-like sequence; BAD1, *Blastomyces* adhesin 1; GMP, Good Manufacturing Practice; GXM, glucuronoxylomannann; IFN-γ, interferon-gamma; IL, interleukin; IND, Investigational New Drug; TNF, tumor necrosis factor.

Competing interests

In the last 12 months, BS has received grant funding from Gilead, Astellas, and Novartis to operate a therapeutic interventional study for the treatment of mucormycosis. He is a shareholder in NovaDigm Therapeutics, which is developing candidal vaccine technology.

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