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Review Article

Aspergillus vaccines: Hardly worth studying or worthy of hard study?

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Abstract

Vaccines rank among the greatest advances in the history of public health. Yet, despite the need, there are no licensed vaccines to protect humans against fungal diseases, including aspergillosis. In this focused review, some of the major scientific and logistical challenges to developing vaccines to protect at-risk individuals against aspergillosis are discussed. Approaches that have shown promise in animal models include vaccines that protect against multiple fungal genera and those that are specifically directed to *Aspergillus*. Advances in proteomics and glycomics have facilitated identification of candidate antigens for use in subunit vaccines. Novel adjuvants and delivery systems are becoming available that can skew vaccine responses toward those associated with protection. Immunotherapy consisting of adoptive transfer of *Aspergillus*-specific T cells to allogeneic hematopoietic transplant recipients has advanced to human testing but is technically difficult and of unproven benefit. While progress has been impressive, much work still needs to be done if vaccines against aspergillosis are to become a reality.

Key words: aspergillosis, fungal vaccines, immunotherapy, allergy, allogeneic hematopoietic transplant.

Introduction

Aspergillus is ubiquitous in the environment; it is estimated that individuals typically inhale hundreds of conidia a day.[1] In the vast majority of individuals, host defenses are able to easily contain the fungus, and the near constant encounters with the fungus are harmless occurrences. However, *Aspergillus* causes a wide spectrum of acute and chronic diseases in persons with compromised host defenses. Thus, in persons with severe immunocompromise, particularly those with impaired neutrophil function and recipients of allogeneic transplants, inhaled conidia can germinate into hyphae, which then invade lung tissue and eventually disseminate to other organs. The burden of invasive aspergillosis (IA) is estimated at over 200,000 persons per year.[2] In patients who are atopic or have cystic fibrosis, sensitization to *Aspergillus* can result in allergic manifestations including severe asthma with fungal sensitization (SAFS) and allergic bronchopulmonary aspergillosis (ABPA).[3,4] An estimated 5 million people have ABPA.[5] Persons with preexisting cavitary lung disease, such as might occur due to tuberculosis or sarcoidosis, are at risk for developing colonization of the cavities with *Aspergillus*. While these so-called

aspergillomas are often asymptomatic, life-threatening complications, particularly hemoptysis, can occur.

Hundreds of species of *Aspergillus* have been described although the vast majority of human infections are caused by a handful of species including *A. fumigatus*, *A. flavus*, and *A. niger*. Many of the antifungals in clinical use lack intrinsic activity against *Aspergillus*, and for those that have anti-mould activity, resistance has become a widespread concern.[6] Tests to diagnose invasive aspergillosis exist, but they generally lack sensitivity; this can result in fatal delays in diagnosis. While the substantial morbidity and mortality associated with aspergillosis have prompted urgent calls for new drugs and diagnostics, they have also stimulated research into vaccines to protect at risk patients and to treat those with disease. Here, I review the obstacles to vaccine development (and their potential solutions), define populations that could benefit from vaccinations, and provide examples of promising approaches. Due to space limitations, many excellent studies could not be included. A comprehensive review of *Aspergillus* vaccines including immunotherapeutic approaches has recently been published.[7]

Are *Aspergillus* vaccines feasible?

As noted above, a large number of individuals are at risk for developing aspergillosis and thus could presumably benefit from vaccination. In this section, the numerous factors which hinder the development, testing and marketing of *Aspergillus* vaccines are discussed along with possible solutions (Table 1). Patients with aspergillosis have a wide range of immunological abnormalities. Those at risk for invasive aspergillosis generally have severe immunocompromise that greatly limits their response to vaccination. This includes defects in innate (particularly neutropenia during periods of chemotherapy) and adaptive defenses. Possible solutions include novel vaccine formulations containing potent adjuvants to elicit protective responses.[8] Patients who will be immunosuppressed in the future, such as those on transplant waiting lists, could be vaccinated prior to transplant when their immune response is relatively intact. For those receiving allogeneic hematopoietic transplants, protective donor lymphocytes could be transplanted. On the other end of the spectrum, those with allergic manifestations have robust but dysregulated immune responses. In this patient population, a successful vaccine strategy could be to dampen or redirect the nature of the immune response such as by shifting the bias from Th2 to Th1.

Most candidate *Aspergillus* vaccines are being testing in mice. Mice and humans diverged approximately 65 million years ago, and while the basics of the immune system are quite similar, there are key differences.[9] In addition, most studies use inbred mice that lack the genetic diversity of "outbred" humans. Perhaps more importantly, laboratory mice live in filtered cages and thus have no natural exposure to *Aspergillus*. This contrasts with humans who are repeatedly exposed to airborne fungi. Possible solutions include the use of multiple animal models including humanized mice, modeling natural exposure conditions by continuous challenge, and conducting *in vitro* human testing.

Aspergillus species and *Homo sapiens* are both eukaryotes. As such, many potential *Aspergillus* vaccine candidates have significant homology to human proteins and may either not elicit robust immunological responses or could trigger autoimmunity.[10] However, with the human genome sequenced, such proteins can be avoided or the homologous portions excluded from vaccines. Another issue is that fungi extensively glycosylate many of their proteins, particularly the cell wall proteins that might be the most promising vaccine candidates.[11] Moreover, the pattern of glycosylation can vary as a function of fungal species. If protective antibody responses are to the glycan portion of the molecule, then expressing the protein in a prokaryotic vector or even a yeast vector would not be expected to work.

Table 1. Obstacles to *Aspergillus* vaccine development.

Obstacle to Vaccine development	Potential solution(s)
Severe immunocompromise	Improved adjuvants
	Vaccine prior to anticipated immunosuppression
	Target arms of the immune system least affected
	Infuse donor lymphocytes
Dysregulated (allergic) responses	Shift immune response to protective
Translation of animal studies to humans	Use multiple animal models
	In vitro and phase one human studies
Fungi and humans are eukaryotes	Avoid homologous protein sequences to minimize the risk of autoimmunity
Aspergillus glycosylates many of its proteins	Use native protein for vaccines designed to stimulate antibody protection
Necessity for large number of patients	Perform adequately powered clinical trials
Commercialization	Attract interest from NGOs
	Form biopharmaceutical companies

One option is to incorporate native glycoprotein into the vaccine.

Criteria for diagnosing allergic manifestations of aspergillosis and subsequent responses to treatment are fairly well established.[12] However, establishing a diagnosis of invasive aspergillosis can be difficult due to the insensitivity of diagnostic tests. As a consequence, the majority of patients enrolled in clinical trials have possible or probable invasive aspergillosis, rather than proven disease.[13] This, combined with the increasing use of anti-mould prophylaxis in high risk groups will necessitate recruiting large numbers of patients for *Aspergillus* vaccine trials. Nevertheless, numerous comparative studies of antifungal regimens for invasive aspergillosis have been successfully conducted, and similar studies with clinically meaningful endpoints could be performed for vaccines. Moreover, improvements in diagnostics could facilitate identification of probable and proven cases of aspergillosis.

Even if preclinical studies result in promising candidates, companies may be reluctant to enter the *Aspergillus* vaccine market due to the large expense of clinical trials and fears that profit margins will be reduced because much of the target population resides in resource-limited areas. Commercial development may require funding from nongovernmental organizations or for investigators to foster development by establishing biopharmaceutical companies. As an example, the company AlerGenetica is focused on vaccines to treat persons allergic to filamentous fungi. Finally, while this review focuses on the development of human vaccines, birds are particularly susceptible to pulmonary aspergillosis. Epidemic and endemic infections at commercial poultry plants, particularly turkey farms, have prompted research into vaccines to protect the birds.[14–16]

Aspergillus vaccines

Pan-fungal vaccines

Aspergillus vaccines can be subdivided into four somewhat overlapping categories: pan-fungal, crude, subunit, and therapeutic. Representative examples of each of these categories will be discussed as well as considerations regarding delivery systems and adjuvants. Pan-fungal vaccines take advantage of shared antigens possessed by fungi to protect against many medically important fungal species genera.[17] Such vaccines may be more likely to attract commercial interest because, if successful, they will protect against many different mycoses. The Cassone laboratory conjugated β-1,3-D-glucan (in the form of laminarin) to diphtheria toxoid. Mice immunized with the conjugate glycoprotein developed robust antibody responses to β-1,3-D-glucan and were protected against challenge with several different fungi including *Aspergillus*.[18] Similarly, Clemons et al. were able to protect mice from five genera of fungi including the *fumigatus* species of *Aspergillus* by vaccination with heat-killed *Saccharomyces cerevisiae* yeast.[17] Protection did not appear to be antibody-dependent. One possible mechanism is stimulation of memory innate defenses (also known as "trained immunity"); β-1,3-D-glucans have been shown to induce epigenetic reprogramming and nonspecific protection against infections.[19] Wuthrich et al. discovered a T-cell epitope in the protein calnexin that was highly conserved across members of the Ascomycota (the fungal phylum that contains *Aspergillus*).[20] The investigators demonstrated that calnexin localized to the surface of the fungi and that vaccination of mice with calnexin resulted in expansion of antigen-specific $CD4^+$ T cells. However, calnexin-mediated protection against *Aspergillus* was not studied. Proteomic approaches have also been used to identify proteins that are abundant in medically important fungi, including *Aspergillus*, without having significant homology to human proteins.[10]

Whole organism and other "crude" vaccines

Crude vaccines consisting of whole *A. fumigatus* (live or killed) or fractions derived from culture filtrates have been studied in mouse models of aspergillosis. Such studies provide a proof of principle. However, this type of approach may not reach human testing due to concerns about possible autoimmune responses and excess reactogenicity to the large numbers of antigens contained in such vaccines. Cenci et al. vaccinated mice by three intranasal inhalations with live *A. fumigatus*, heat-killed *A. fumigatus*, or a crude culture filtrate.[21] They then immunosuppressed the mice with cyclophosphamide and challenged them with intranasal or intravenous *A. fumigatus* conidia. While survival was not prolonged in the mice that received heat-killed fungi, significant protection was observed in the mice that received live *A. fumigatus* or the crude filtrate. $CD4^+$ T cells, IFN-γ, and IL-2 were required for protection. In contrast, mice that lacked IL-4 were more resistant to *A. fumigatus* challenge.[22]

Subsequent studies demonstrated that immunization of mice with live (but not heat-killed) conidia induced IFN-γ-producing, *Aspergillus*-specific $CD4^+$ T cell and humoral responses.[23] In contrast, the heat-killed conidia induced $CD4^+$ T cells that produced IL-4 and IL-13. The mechanism responsible for the disparate responses to heat-killed and live conidia remain speculative but could be related to some of the live conidia germinating into hyphae, which is the morphotype seen in invasive disease. Hyphae express a unique set of antigens and stimulate different pulmonary dendritic cell responses compared with conidia.[24]

Subunit vaccines and monoclonal antibodies

A wide variety of *Aspergillus* subunit vaccines, defined as vaccines containing one or more purified components, has been tested in preclinical models. Recombinant *Aspergillus* proteins, which elicit vaccine-mediated protection in models of aspergillosis, include Asp f3, Gel1, Asp f9 (Crf1), Asp f16, and Pep1.[25,26] The mechanism of protection has been presumed to be $CD4^+$ T cells although in one study adoptive transfer of cytotoxic T lymphocytes specific for Asp f16 pepides extended survival in mice infected with *A. fumigatus*.[27] Purified cell wall glycans also have been used as immunogens. Protection was observed following intranasal vaccination with α- and β-1,3-D-glucans but not with galactomannan.[26] As T cells generally recognize peptides and not carbohydrates, it is likely that protection is antibody-mediated. Finally, mice vaccinated with cell wall mannans were protected from a challenge with *A. fumigatus* conidia, with enhanced protection seen if the mannan was conjugated to bovine serum albumin.[28]

Passive administration of a monoclonal antibody directed against cell surface *A. fumigatus* antigens extended the median survival of mice that received a lethal fungal challenge.[29] In addition to raising the possibility that prophylactic administration of monoclonal antibodies could be given to high-risk patients, this study provides a proof of principle that vaccine-elicited antibodies can be protective. Although antibodies traditionally were thought to protect against mycoses by promoting opsonization and complement deposition, this antibody as well as another monoclonal antibody directed against cell wall catalase B had direct inhibitory activity against *A. fumigatus*. [29,30]

Therapeutic vaccines

In patients who receive allogeneic hematopoietic transplants, most cases of invasive aspergillosis occur after neutrophil recovery, suggesting the importance of T-cell defenses, which are considerably slower to reconstitute. This creates a unique situation in which donor T cells can be expanded with antigens *ex vivo* and then adoptively transferred into the patient. In mouse studies, adoptive transfer of *Aspergillus*-specific $CD4^+$ T cells extended the median survival of mice with invasive aspergillosis receiving allogeneic bone marrow transplant.[31] This study provided the foundation for a study in humans with invasive aspergillosis following haploidentical hematopoietic transplantation.[32] Donor $CD4^+$ and IFN-γ-producing T cell clones specific for *Aspergillus* antigens were generated and expanded by incubating peripheral blood mononuclear cells with heat-killed conidia. Transplant recipients who developed evidence of invasive aspergillosis received adoptive T cells. Remarkably, 9 of 10 patients who received such immunotherapy resolved their infection. In contrast, resolution was seen in only 6 of 13 control patients who did not receive T cells.

A more rapid technique for T-cell expansion based on activation-dependent expression of CD154 and CD137 following incubation of peripheral blood mononuclear cells with recombinant *A. fumigatus* proteins was recently described although has yet to be tested on humans.[33] Importantly, the generated T-cell lines showed high IFN-γ and IL-17 responses to fungal species from a wide range of medically important genera, including *Aspergillus*, *Candida*, *Fusarium*, *Mucorales*, and *Scedosporium*. The efficiency and efficacy of the process could be increased by the identification of additional antigens that stimulate human $CD4^+$ T cells[34,35] and by the simultaneous expansion of $CD8^+$ T cell clones. Recently, a highly innovative technique to generate T cells reactive with *Aspergillus* and other fungal antigens was developed in which human T cells were genetically modified to express the B-glucan recognition receptor Dectin-1.[36] Such bioengineered T cells avidly bound to and killed *A. fumigatus* germlings.

Delivery systems and adjuvants

Purified antigens tend not to elicit strong immune responses unless they are administered with stimulatory adjuvants and/or delivery systems.[8,37] For carbohydrate antigens such as β-1,3-D-glucan that are poorly immunogenic, conjugation with a protein carrier greatly boosts specific antibody responses.[18] The adjuvant or delivery system used informs the nature of the immune response. Alum, the adjuvant used in most commercial vaccines, tends to bias toward antibody and Th2 cell responses. Bozza et al. were able to induce protective Th1-mediated responses using a vaccine consisting of dendritic cells pulsed with live *A. fumigatus* or *A. fumigatus* RNA.[31] Another approach used dendritic cells transduced with an adenovirus vector encoding IL-12.[38] Mice receiving DCs pulsed with heat-killed *A. fumigatus* had greater survival and lower fungal burdens.

Dendritic cell vaccines are probably too labor intensive and expensive for routine vaccination of a large population but they do have translational potential in therapeutic vaccines. Other studies successfully adjuvanted *Aspergillus* vaccines with unmethylated CpG-rich oligonucleotides (which are ligands for TLR9) and TiterMax.[25,26,39] Interestingly, some candidate antigens, such as Asp f3 and Asp f9, have IgE binding epitopes and have been identified as allergens.[7] However, when given with the proper adjuvant, they induce protective responses in mice.[25] This raises the prospect that adjuvanted vaccines could be used in patients with allergic aspergillosis to convert deleterious atopic responses to ones that are more balanced.

Conclusions

Vaccination is arguably one of the greatest public health successes in the history of medicine. Nevertheless, effective fungal vaccines for clinical use have remained elusive; this despite the burgeoning numbers of persons with aspergillosis and other life-threatening fungal diseases.[2] Advances in understanding the complex ways the immune system controls *Aspergillus* and what goes awry in immunocompromised populations have informed strategies for preventive vaccine development. Similarly, numerous immunogenic antigens have been identified at the molecular level, which could serve as candidate antigens in human vaccines. Novel adjuvants and delivery systems are becoming available that can skew vaccine responses toward those associated with protection. In a pilot trial, immunotherapy demonstrated great potential in allogenic hematopoietic transplant recipients with invasive aspergillosis; new technologies for expanding T cells promise to make this procedure safer and more efficacious. Despite the impressive progress, much work still needs to be done if vaccines against aspergillosis are to become a reality. Given the substantial morbidity and mortality associated with aspergillosis, it is truly a task worthy of hard study.

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Declaration of interest

The author reports no conflicts of interest. The author alone is responsible for the content and the writing of the paper.

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