

Review Article

Developing a vaccine against aspergillosis

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Although there is considerable experimental data to support the idea, bringing a fungal vaccine to fruition has been elusive. Moreover, vaccinating the immunocompromised, susceptible to an opportunistic disease such as invasive aspergillosis, seems formidable. However, pioneering studies using *Aspergillus* particulate forms or homogenates, and recently, recombinant proteins, have demonstrated feasibility. Moreover, T cell receptors also recognize glycotopes if presented in the appropriate MHC-binding context. The potential role of induced antibody has been appreciated only recently. Recent studies in our laboratory with heat-killed *Saccharomyces* (HKY) have raised the possibility of development of a panfungal vaccine. This yeast may be nature's experimental reagent, to show the way to a protective protein-carbohydrate conjugate vaccine. Subcutaneous HKY is an effective vaccine against *Aspergillus*, *Coccidioides* or *Candida* challenge. We have learned the protective moiety is in the cell wall, and proteins, glucan and lipid all seem important. We have also found the cell wall glycans alone, mannan or glucan, as a vaccine each provide significant protection. This leads to consideration of the importance of glycosylated proteins and glycan polymer-protein conjugates in vaccine development. We think the most productive route to a fungal-specific vaccine may be a conjugate vaccine that combines the optimally configured glycan with a specific immunogenic protein. Our work so far suggests that some proteins may be sufficiently cross-immunogenic, such that combined with the appropriate glycan, it may be possible to develop a pan-fungal vaccine.

Keywords Aspergillosis, vaccines, *Aspergillus fumigatus*, glycans, proteins

Introduction

A review article [1] published in 1967 cited 32 articles on fungal vaccines that had demonstrated induction of increased resistance, yet today there are only two commercial fungal vaccines: Russian and Czech veterinary vaccines against ringworm. However, the tide of scientific discovery, such as the mapping of the pathogen genomes, definition of fungal antigens, and improved understanding of fungal immunology and of pathogen virulence factors, as reviewed at the Advances Against *Aspergillus*

conferences and other meetings, has brought clinical fungal vaccines to the realm of possibility. The present review will extend the data base from an earlier review [2], and that of the recent update at the Advances Against Aspergillosis conference [3].

The task

Vaccinating the immunocompromised population susceptible to an opportunistic disease such as invasive aspergillosis would seem a difficult task, but there is some 'low-hanging fruit' that might be considered, as an initial step, as the least immunocompromised, most promising, candidates, such as chronic granulomatous disease patients, transplant candidates prior to transplant, leukemics after successful induction therapy, solid tumor patients at diagnosis, patients with

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rheumatic or inflammatory bowel disease before immunosuppression, and intensive care unit patients in the high risk groups for aspergillosis but who lack the classical risk factors such as steroids and cytotoxic chemotherapy. Another approach would be to immunize the donors of hematopoietic stem cell transplants. Encouraging evidence has been provided that CD4-deficient hosts can be successfully vaccinated, and in them CD8 cells can substitute to develop vaccine-induced immunity [4–6]. Moreover, prevention of disease may not be required for a vaccine to be successful; a vaccine could be useful if it augmented an impaired immune response, ‘held the fort’ until immunity recovered (immunosuppression reduced, or neutrophils return), or acted synergistically with antifungal therapy.

Pioneer studies

The initial thrust in developing an *Aspergillus* vaccine utilized particulate forms of the fungus, or homogenates of them, and most recently, recombinant proteins. The aim in most studies was to recruit memory T cells that, on infection, would generate a Th1 proinflammatory response. This response would produce cytokines that would upregulate phagocyte function, or possibly direct T cell killing [7]. Dendritic cells were shown to be an important intermediary in some of these studies [8–10]. Most important, some of these studies showed protection even in neutropenic, marrow transplant, or steroid-treated animals [8,11–14].

For examples of these approaches, germlings have been shown to be protective in turkeys [15], conidia have been shown to produce protective responses [9,14,16–19], as have recombinant protein antigens [8,11,12,17,20,21]. In general, *Aspergillus* hyphal extracts have not been as efficacious for vaccine protection. A live, attenuated organism has also been shown to be protective [22]. Notably, one study [20] demonstrated the recombinant protein antigen was only protective if presented in particulate form or with an adjuvant. Protection has been demonstrated using the subcutaneous route for vaccine delivery [9,13], and this was even shown to be superior to the intranasal route [13]. In aggregate, these studies suggested the limb of immunity stimulated was cell-mediated immunity [3,9,19,20].

Antibody

Later studies raised the possibility that antibody could be an important component of antifungal, and specifically anti-*Aspergillus*, protection. The early studies did not define what the protective antibody was directed against [23] or showed activity against an *Aspergillus* peptide or protein [24].

Several studies in the *Candida* field showed immunization with whole *Candida* or purified glycans could produce antibodies inhibitory to *Candida in vitro* or *in vivo* [25–27]. Some notable studies emphasized that an antibody against glucan could be produced [28,29], and it was shown this antibody could also inhibit *Aspergillus* hyphae *in vitro*, and contributed to protection against *Aspergillus* challenge [29].

In this context, a prominent vaccine authority has suggested an overview that antibody may be thought of as a correlate of protection against infection, whereas T cell responses may be thought of as a correlate of protection against disease (once infection is established) [30]. Although a prevailing thought had been that cell-mediated immunity was key to defense against fungi [31], there are a number of ways in which antibodies could be useful in combating fungal infections: activation of complement, with neutralization of virulence traits; opsonization, affecting the direction and vigor of cell-mediated (particularly T cell) immunity to directly inhibit fungal growth, inhibit adherence and inhibit germination [24,25,27,32–34]. Antibody affinity, specificity, and time of appearance or administration would be key to any antibody effect [35].

Heat-killed *Saccharomyces* as a vaccine

Recent studies in our laboratory with heat-killed *Saccharomyces* have raised the possibility of the development of a panfungal vaccine. This yeast may be nature’s experimental reagent, to show the way to a protective protein-carbohydrate conjugate vaccine.

Our path began with vaccine studies to utilize *Saccharomyces cerevisiae* as a recombinant delivery system for cloned *Aspergillus* protein antigens. This strategy is based on observations that such yeasts stimulate innate CD8 reactions [36], and uptake of yeasts by dendritic cells leads to increased expression of CD40, IL-12, co-stimulatory molecules and increased MHC I- and II-restricted T cell (especially CD8) responses [37]. Although we found that *Saccharomyces* expressing *Aspergillus* hemolysin (a candidate *Aspergillus* immunodominant antigen) induced protection, to our surprise, we found *Saccharomyces* without the plasmid vector containing the *Aspergillus* gene, or with an empty plasmid vector, gave just as much protection against *Aspergillus* challenge in mice [Capilla J, Clemons KV, Miller TK, Selitrennikoff CP, Stevens DA. *Aspergillus fumigatus* antigens and fungal elements as vaccines against aspergillosis in mice. 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, 2006, Abstracts, no. G-153]. This observation (with heat-killed *Saccharomyces* alone) was later reaffirmed in many experiments, and optimal regimens defined [Capilla J, Clemons KV, Stevens DA. The friend of man a friend

again?: *Saccharomyces* as a vaccine against invasive aspergillosis. 47th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, 2007, Abstracts, no. G-1708]. As developing a vaccine against *Coccidioides* is a long-standing interest of our laboratory, we had the opportunity to also study heat-killed *Saccharomyces* as a vaccine against a primary pathogen. In comparative studies with the gold standard vaccine preparation in the *Coccidioides* field, formalin-killed spherules, we showed equivalent protection with heat-killed *Saccharomyces* and the heat-killed *Saccharomyces* was superior to a well-described coccidioidal immunogen, PRA/Ag2, given with adjuvant. Most exciting, we were able to obtain protection with *Saccharomyces* by the oral route. These studies have been published in full [38]. More recently, we also reproducibly found heat-killed *Saccharomyces* can protect mice against a systemic *Candida* challenge [Liu M, Clemons KV, Stevens DA, unpublished results].

In ongoing studies, we are investigating the nature of the protective moieties in heat-killed *Saccharomyces*. Separation of the pellets from the supernatant materials in fractionation studies, disruption of the proteins by protease digestion or extreme heat treatment, and killing the yeasts by instead breaking them up with glass beads or treating with formalin have been studied, as well as (in collaboration with Gary Ostroff, Stuart Levitz and Charles Specht, University of Massachusetts) increasing alkali treatments, or alkali plus organic solvent treatment. We have learned that the protective activity always remains in the pellet, but the ablation of the protection with protease, preservation after alkali treatment, and ablation with organic solvent suggest the importance of proteins, glucan and lipid, respectively. Thus, the cell walls may have immunostimulating or adjuvant properties, or may be a delivery vehicle for cell wall-associated antigens. The similar protection apparently afforded by either cell wall or whole yeasts is of interest, in view of immunological differences described for whole yeasts and yeast hulls [39]. Our use of heat to kill the yeast may have been fortuitous, in that heating yeasts increases the binding of β -glucan by dectin-1 [40], at least in part by increasing exposure of binding sites [41], and further, heat denaturation of proteins tends to shift the host response against them to a Th1 type [42]. In addition, *Saccharomyces* has double stranded RNA, from ubiquitous fungal viruses [43] and fungi also have CpG DNA, a known immunoadjuvant. However, our experience with cell pellet protection may suggest these nucleic acid adjuvants are not essential to the protection we are seeing. In collaboration with Markus Kalkum and team (at City of Hope), we have looked for proteins that could explain the cross-protection induced by *Saccharomyces* against aspergillosis or coccidioidomycosis. Using two-dimensional Western blotting

and sera from mice immunized with *Aspergillus* hyphal sonicate or heat-killed *Saccharomyces*, or coccidioidal-infected rabbits, we found common reactivity against several proteins, particularly an immunodominant 75 kD common protein in *Saccharomyces* extract, *Aspergillus* hyphal sonicate, or *Aspergillus* culture filtrate. This has been identified by mass spectrometry in *Aspergillus* as a dipeptidyl peptidase [44], previously suggested as a vaccine candidate. However, there are other homologous proteins already described between *Saccharomyces*, *Aspergillus*, and *Coccidioides*. These include chitinase cts2 in *Coccidioides* [45], cts1 chitinase in *Saccharomyces* [46,47], and chitinases of *Aspergillus* [48]; AspF3 in *Aspergillus*, Pmp1 in *Coccidioides*, and Ahp1 in *Saccharomyces* [20]; and cell wall proteins Gel1p in *Aspergillus* and *Coccidioides*, with Gas1p in *Saccharomyces*, and between Crh1 in *Saccharomyces* and Crf1p in *Aspergillus* [8]. Gas1p and Crh1 have already been suggested as possibly protective against aspergillosis [8]. It is highly likely that many other proteins with homology among these fungal species will be described in future publications and some may be found to be strong vaccine candidates. For example, in ongoing work, we and our collaborators have already preliminarily identified that 7 of the 20 most abundant proteins of *A. fumigatus* and *Coccidioides posadasii* hyphae share >50% sequence identity.

In the course of our studies we have already learned, presented only in abstract form here, that the protection is not *Saccharomyces cerevisiae* strain-specific, nor even *Saccharomyces* species-specific, not mouse strain-specific, and not related to *Saccharomyces* in the diet of the test mice; heat-killed *Saccharomyces* provides as potent protection against aspergillosis challenge as heat-killed *Aspergillus* conidia or hyphae, alum potentiates the protection induced by heat-killed *Saccharomyces* against aspergillosis, but not against coccidioidomycosis; that antibodies to glucan and mannan are made after heat-killed *Saccharomyces* vaccination, and that spleen cells and lymph node cells of vaccinees proliferate in response to vaccination (CD3 and CD8 lymphocytes) and produce proinflammatory cytokines, and that proinflammatory cytokines are found in the bronchoalveolar lavage fluid of vaccinated mice [Liu M, Clemons K, Bigos M, Stevens, D. Immune responses induced by heat killed yeast, a vaccine against aspergillosis in mice. Program of the 4th Advances Against Aspergillosis meeting, Rome, Italy, 2010, abstract no. 20; Liu M, Clemons K, Stevens D. *Saccharomyces* as a vaccine against invasive aspergillosis is enhanced by alum but is not mouse strain or *Saccharomyces* species specific. Program of the 4th Advances Against Aspergillosis meeting, Rome, Italy, 2010, abstract no. 21; Johansen M, Alvarado D, Liu M, Clemons K, Stevens D. Efficacy of different strains of *Saccharomyces cerevisiae* as a vaccine against

systemic aspergillosis. Program of the 4th Advances Against Aspergillosis meeting, Rome, Italy, 2010, abstract no. 22: Johansen M, Liu M, Clemons K, Stevens D. Does the mouse chow influence the effectiveness of a heat-killed *Saccharomyces* vaccine against systemic aspergillosis? Program of the 4th Advances Against Aspergillosis meeting, Rome, Italy, 2010, abstract no. 88]. We (in collaboration with Slavomir Bystricky and team in Bratislava) have also found that the cell wall glycans alone, mannan or glucan, as a vaccine each provide significant protection against *Coccidioides* or *Aspergillus* challenge, which is greatly enhanced by conjugation to a protein, bovine serum albumin, and that anti-mannan antibody is protective against aspergillosis [Liu M, Machova E, Nescakova Z, Clemons KV, Martinez M, Chen V, Bystricky S, Stevens DA. Vaccination with mannan protects mice against invasive aspergillosis. 49th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, 2009, Abstracts, no. G1-892.]. The observation of mannan protection is of interest, in that mannan from *Candida* was used for our immunization, and fungal mannans are more diverse among fungal species than glucan. It is of considerable interest that subcutaneous administration of heat-killed yeast did not exhibit dose-limiting toxicity in phase I human clinical trials [49].

Glycans

Although carbohydrates produce T-independent immune responses, favoring humoral immunity, which do not lead to isotype switching and lack memory, there are exceptions [50,51], and T-cell receptors are also able to recognize glycotopes if they are presented in the appropriate MHC-binding context [52]. Coupling to proteins converts glycotopes into complexes that as a result of antigen processing result in the production of T-reactive peptides, that when presented to T cells with MHC elicit help, result in T-dependent responses, and antibodies against carbohydrates with memory cells [25,52,53]. The success of conjugated vaccines in the bacterial field [50,54], evidence that polysaccharide-protein conjugate vaccines can be effective against cryptococcosis and candidiasis [55,56], the lack of robust anti-coccidioidal immunity after immunization with cloned coccidioidal proteins given alone or with an adjuvant in comparison to whole killed coccidioidal spherules [57–59], particularly when the challenge is given by the natural, respiratory, route [60], leads to the consideration of the importance of glycans, glycosylated proteins and glycan polymer-protein conjugates in further vaccine development [32].

In conjugating protein to glycans, how the protein and carbohydrate are coupled is a key step. The carbohydrate can be destroyed [25], there can be undesirable intra- and

inter-chain crosslinking of the polysaccharide, or several polysaccharide chains can attach to a protein molecule at multiple sites, resulting in high molecular weight aggregates, which is undesirable. Coupling methods that have been successful include triethylene glycol acrylate and 1-cyano-4-dimethylaminopyridium tetrafluoroborate (CDAP) [25,53,61]. CDAP is less hazardous, easier to use, and more rapidly active than a previously-used agent, cyanogen bromide, has a more flexible pH range, and can react with more diverse types of polysaccharides [53]. CDAP activates the carbohydrates at the hydroxyl sites to enable binding, and the resultant cyanyl ester groups react with amine groups on the protein. The resultant 3-dimensional configuration of the conjugate, and exposure of particulate epitopes, becomes important in recognition [62].

β -glucan is a pleiotropic immunomodulator [32,63–68], binding to the Dectin-1 receptor. It has been shown to stimulate immunity to bacteria, viruses, parasites, and fungi; increase phagocytosis and antimicrobial killing, prime spleen cells for cytokine production, increase natural killer cell activity, increase co-stimulatory molecules, activate dendritic cells and stimulate dendritic cell maturation, and increase matrix metalloproteinase-9. It also stimulates hematopoiesis, increases hematopoietic recovery after injury, and mobilizes stem cells to the periphery. Particulate material is more potent than soluble, and the molecular weight and type of branching are critical to the immunologic effects [69]. It has been shown to be an adjuvant (including to fungal antigens), and to have inflammatory (in which case antibodies to it could immunomodulate by an anti-inflammatory action) or anti-inflammatory properties.

Aspergillus and *Candida* appear to have more shared glucan epitopes than with more distant fungi, such as mushrooms [70]. Another group has previously reported β -glucan can be protective against aspergillosis, and stimulates a Th17/Th1 profile [8].

Fungi, as other eukaryotes, glycosylate proteins, and tend to use mannose for this purpose. The mannan on the protein can direct the complex to the host dendritic cell mannose receptor. O-linked mannosylation particularly stimulates the immunogenicity of proteins [71]. Mannan is also a pleiotropic immunomodulator, binding to the mannose receptor, Toll-like receptors, and others [66]. Conjugates to protein, or presentation in liposomes, have been found to be protective vaccines against candidiasis [26,61], transferable by serum [61]. This is of interest because mannan has been shown to decrease the neutrophil respiratory burst, myeloperoxidase release and killing of *Candida*, and to decrease lymphoproliferative responses and block immune or protective responses [28,72–74]. Again, the length of the mannan polymer is a critical factor in determining the direction and effect of the immunomodulation.

The structure and composition of mannan is species-specific, in the substitution frequencies and side chains formed [27]. The key mannan in *Aspergillus* is galactomannan. Oligosaccharides can be synthesized to mimic mannan epitopes in native glycoproteins [75].

Conclusion

In combination of themes developed in this review, investigators have demonstrated that a glycan (mannose trisaccharide)-protein (from the *Candida* cell wall) yielded an antibody response to both components, and provided a protective response against candidiasis [61], though the choice of protein was critical for whether a protective response was elicited. Indeed it has been suggested it is '... attractive to consider the possibility of designing protein-conjugate vaccines that elicit protective antibodies to both moieties...' [32].

What does this suggest for the future? We think the most productive route to a fungal-specific vaccine may be to work towards a conjugate vaccine that combines the optimally configured glycan with a specific immunogenic protein. However, our work so far suggests that some proteins may be sufficiently cross-immunogenic, such that combined with the appropriate glycan, it may be possible to develop a pan-fungal vaccine.

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