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Prospects for the Development of Fungal Vaccines

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INTRODUCTION

In an era of our medical history that is punctuated by the term "cost-effective," immunization is incontrovertibly a paradigm of a utilitarian medical substance—the greatest good for the greatest number. Moreover, immunization provides this effect at the lowest cost to the public. Several factors have contributed to the spiraling interest in vaccines. First, they produced the successful worldwide eradication of smallpox and the virtual elimination of poliomyelitis from developed nations. Second, they have effectively limited the spread of many childhood and adult diseases including measles, mumps, rubella, hepatitis B, and *Haemophilus influenzae* type b infections. Third, the emergence of viruses, bacteria, and fungi that manifest high-level resistance to antimicrobial agents has prompted medical specialists to reconsider the importance of preventive measures. Fourth, the escalating advances in biotechnology offer the hope for new and more effective vaccines. The concatenation of these milestones has led to the reemergence of vaccines as the leading edge of medical therapy.

In this review, I will summarize the recent advances made in the identification and generation of possible vaccine candidates for medically important fungi. Much of the work prior to the 1980s will not be discussed, and readers interested in the historical perspective are referred to two excellent comprehensive reviews (56, 90). The reader should note that although vaccination and immunization often are used interchangeably, they have, strictly speaking, different meanings. Vaccination does not necessarily produce immunization. The two words are synonymous when vaccination leads to immunization against infection. The term "immunization" is used herein when it is clear that a vaccine induces a protective response.

HISTORICAL PERSPECTIVE

The idea that disease could be obviated by vaccination was acted upon in China and India before 1,000 B.C., when material from smallpox pustules was intranasally instilled into humans. In the 18th century, Edward Jenner observed that milkmaids exposed to cowpox did not contract smallpox. To test his hypothesis that cowpox could prevent smallpox infection, he used a naturally attenuated strain of cowpox to vaccinate an 8-year-old boy; 2 months later, he exposed the boy to smallpox. The youth was protected from subsequent infection. Although the results were disparaged originally, they spawned a new era in the prevention of infectious diseases (19, 57).

Since the original pioneering work of Jenner and Pasteur,

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many vaccines have been licensed for viral or bacterial diseases of humans but none have been licensed for medically important fungi. The largest clinical trial of a vaccine for a mycosis, specifically coccidioidomycosis, was performed in the early to middle 1980s by the Valley Fever Vaccine Study Group (83). Nearly 3,000 subjects who were skin test negative for this fungal infection were randomized blindly to receive either whole spherules killed with formaldehyde (1.75 mg of spherules) or saline. During 5 years of observation, 12 cases of coccidioidomycosis developed in the placebo controls whereas 9 cases were detected in the vaccine recipients. This slight difference was statistically insignificant. One of the problems with the potential effectiveness of the spherule preparation was that less than 30% of the vaccinated subjects manifested evidence of a response to the spherule preparation. Thus, it is possible that in almost 70% of subjects, the vaccine was not immunogenic.

Despite the failure of the vaccine for coccidioidomycosis, there are useful fungal vaccines in veterinary medicine. Equines and other mammals can be infected with *Pythia insidiosum*, which causes a debilitating cutaneous, subcutaneous, or systemic disease (20). Two vaccines, Miller's (68) and Mendoza's (67), have been demonstrated to be of use to both prevent and treat horses infected with *P. insidiosum*. The former is composed of sonicated hyphal antigens, whereas the latter is prepared from culture filtrate antigens (67, 68). Specific immunogens from either preparation have not yet been identified; however, work is ongoing to determine if specific constituents of the extracts can mimic the protective activity of the extract. These results provide optimism that a fungal vaccine for humans can be created, although the hurdles are great.

The concept of fungal vaccination for humans remains viable but has not attracted much attention because of the relatively low incidence of infection and the limited geographic distribution of several fungi compared to many viral and bacterial diseases. However, the advent of AIDS and the use of increasingly potent immunosuppressive therapies to combat autoimmune diseases, malignancies, and transplantation rejection have created a revitalized interest in the medically important fungi. Fungal diseases are no longer merely arcane infections; they have gained international recognition as important causes of morbidity and mortality. Accordingly, investigators have demonstrated a growing interest in fungal vaccines.

GOALS OF IMMUNIZATION

Before embarking on a review of the means by which vaccination against mycological agents is being conducted, it is important to determine what are the expectations for an effective fungal vaccine. First and foremost, vaccines against medically important fungi that are found in the environment should be capable of promoting the capacity of the immune system to sterilize tissues. Otherwise, the vaccines do not provide any advantage over natural host defenses that usually can limit the invasiveness of the infecting fungus but do not necessarily sterilize tissues. The human host is most often successful in limiting the spread of *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Blastomyces dermatitidis*, and *Coccidioides immitis* within tissues, but a fraction of the infecting inoculum may survive for years. These niduses of infection may serve as reservoirs for reactivation if the host immune system becomes impaired. Thus, any vaccine against fungi must prevent the establishment of a dormant state in the host. This requirement is not substantially different from that for potential vaccines against human herpesviruses or hepatitis B virus which become latent. Alternatively, a useful vaccine in individuals who have been infected by fungi that establish a dormant state would be one that protects against reactivation. It is possible that one putative immunogen functions to prevent both new infections and reactivation of latent foci. The likelihood that the immunogen might exert both activities would be critically dependent on the mechanisms that the host uses to clear new infections and to maintain the dormant foci. If the cells and cytokines that are involved in these processes are identical or highly similar, it is quite likely that an immunogen will be effective in both conditions. However, if those mechanisms are divergent, the prospect for a single immunogen expressing protective activity against new infections and reactivation disease is limited.

An additional challenge for fungal vaccine development is the identification of an immunologically active substance that can promote the clearance of dormant organisms. Although very little is known about the mechanisms involved in maintenance of the dormant state, a vaccine that could not only arm the host immune system to sterilize tissues but also lead to elimination of persistent fungi would reduce the risk of reactivation disease in immunosuppressed as well as immunocompetent individuals considerably. However, the first goal that must be met is to create a vaccine that can limit the ability of the fungus to establish a latent state.

EPIDEMIOLOGICAL REQUIREMENTS FOR A SUCCESSFUL IMMUNIZATION PROGRAM

In parallel with any large-scale institution of a fungal vaccine program, sound and thorough epidemiological data concerning fungal diseases must be accumulated. Identifying who is at risk for infection and the age at which infection is acquired are two key elements in the development of a vaccine program. Unfortunately, there is little epidemiological information about the fungal diseases. With a few exceptions, fungal diseases do not require reporting to state health departments or the Centers for Disease Control and Prevention. Thus, the extent of active cases of mycoses has been determined based on hospital discharges. However, with the increasing emphasis of medical practice on the outpatient setting, it is likely that hospital information will tremendously underreport many fungal infections.

Another difficulty confronting acquisition of epidemiological data is the lack of suitable reagents for detecting prior exposure to fungal infection. Although histoplasmin, coccidioidin, and spherulin have proven useful in identifying exposed individuals, functional reagents for the other mycoses, especially deep mycoses, are practically nonexistent. For example, *C. neoformans* has a ubiquitous distribution yet the true prevalence of exposed individuals is not known. Cryptococcin was used as a skin test reagent, but its effectiveness in recognizing infected individuals is unclear (74, 88). A similar case can be made for blastomycin. With the rapid advances in molecular biology and protein chemistry in association with an influx of talented investigators, it is anticipated that new diagnostic reagents will be available to unearth the true extent of exposure.

A related issue is the lack of contemporary data. Much of the epidemiological data was collected decades ago. There is reason to believe that such information may not be germane to the current decade or the new millennium. There has been a rapid shift in demographics in many countries as they evolve from agrarian societies to industrialized ones. Thus, exposure to soil-based fungi may not be as frequent as it was in the middle of the 20th century. As an example, previous data indicated that a history of exposure to *H. capsulatum* was present in up to 90% of naval recruits from the southwestern

Ohio region (82). However, in studying people from this region of endemic infection, I have found only about 50% whose peripheral blood mononuclear cells respond to *H. capsulatum* antigens. Although the sample size is quite small (<20 individuals), it is possible that the true prevalence has declined with the change in demographics.

A comprehensive reexamination of the epidemiology of the fungi is critical in establishing the foundation for a vaccination program. Introduction of a fungal vaccine program should be united with a systematic survey of the incidence and prevalence of infection.

MECHANISMS OF PROTECTION

The design of any vaccine, including those for the fungi, must begin with a thorough understanding of the mechanisms that confer protective immunity in the host against a specific pathogen. When that information is available, the search for constituents from the fungus that elicit the particular type of immune response that is necessary for clearance can be undertaken. In this section, I will review the two principal forms of immunity—innate and acquired. Within the category of acquired immunity, both humoral and cell-mediated immunity will be discussed.

Innate Immunity

Innate or natural immunity can be defined as the component of the immune system that utilizes germ line-encoded molecules to eliminate all foreign substances. This system lacks memory and is composed of physical barriers, soluble mediators (e.g., complement), and various cell populations including macrophages, neutrophils, and natural killer (NK) cells. For the purpose of this review, I will focus on the putative influence of specific cell populations in the development of immunity associated with fungal vaccine administration.

Although cellular constituents of innate immunity often are considered the "first-line" of host defenses because of their phagocytic and microbicidal properties, there is mounting evidence that they are crucial in the initiation and amplification of cell-mediated and humoral immunity (33). Polymorphonuclear leukocytes (PMNs) are an important arm of the effector limb of immunity; however, these cells also synthesize and secrete several cytokines including interleukin-1 (IL-1), IL-12, tumor necrosis factor alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage-CSF (M-CSF), which are key elements in the afferent limb of the immune response (8, 33, 63). Although these cells are short-lived (usually <48 h), they may be triggered to release these cytokines by components within a fungal vaccine or an adjuvant. For example, killed *C. neoformans* can induce human neutrophils to produce TNF-α and IL-1β (86). Similarly, killed *Candida albicans* stimulates PMNs to release IL-1, IL-6, and TNF-α (14, 30). Mannoprotein from *C. albicans* activates human PMNs to generate GM-CSF (81). The fact that inert fungal elements or extracts from fungi can activate PMNs to release cytokines suggests that enhancing the production of pivotal cytokines by PMNs may be important in the bioactivity of the vaccine.

Monocytes, macrophages, and NK cells are potent generators of cytokines involved in the differentiation of cellular humoral immune response. Monocytes and macrophages produce numerous cytokines, among which IL-1, IL-10, IL-12, and TNF-α are vital in the initiation and amplification of T-cell and B-cell-dependent immune responses (33). NK cells are capable of producing IL-12, TNF-α, and gamma interferon (IFN-γ) (8, 33, 55). In fact, in murine leishmaniasis, the production of IL-12 and IFN-γ by NK cells during the acute phase of the infection is a critical determinant in the establishment of a protective immune response (85). NK cells release TNF-α after exposure to *C. albicans* (29). Moreover, killed *C. neoformans* can trigger the release of IL-12 and TNF-α by monocytes or macrophages (45, 61). It is reasonable to conclude that the release of cytokines by cells of the innate immune system is central to the evolution of a protective immune response. Thus, the design of vaccines or adjuvants should include elements that can stimulate innate immunity to generate the desired protective response.

Acquired Immunity

In contrast to innate immunity, acquired immunity requires recognition of specific foreign molecules. The magnitude of the response is often amplified upon each exposure to the specific foreign substance. T and B cells are the two principal cellular constituents of acquired immunity, and one of the distinguishing characteristics is that they possess memory or recall for the molecule(s) that initially activated them. Although stimulation of innate immunity by vaccine components may be achieved, it is the humoral and cellular immune systems that must be activated to provide long-lived immunity against fungi. Since many species of fungi trigger both arms of the immune response, the challenge in vaccine design is to determine the relative importance of humoral and cellular immunity in host defenses and to identify fungal antigens that will elicit protective antibodies, protective T cells, or both.

Humoral immunity. Innate and cell-mediated immunity have been acknowledged as the primary mediators of host resistance to the fungi, but there is a growing body of evidence that antibodies are key elements of the protective immune response to some fungi. Early studies in which passively transferred polyclonal antisera from immunized animals failed to confer protection may have been flawed, because it is now clear that polyclonal antisera may be comprised of protective, irrelevant, and harmful antibodies (13). Thus, the effect of a particular antiserum is dependent upon the summation of the biological activities of the three types of antibodies.

The advent of monoclonal antibody technology has facilitated the concept of mapping determinants to identify those that evoke a protective antibody response. Defining the smallest epitope or epitopes that can elicit protective antibodies has the advantage of eliminating unnecessary or unwanted antibodies produced in response to complex antigenic substances. One of the challenges in vaccination is to limit the repertoire of antibodies that are generated in response to an epitope. Studies with passively transferred monoclonal antibodies do not address the issue of whether immunization with a specific epitope can stimulate the production of a limited repertoire of plasma cells that produce only protective antibodies.

Studies with antibodies to *C. albicans* and *C. neoformans* represent the best examples of the powerful influence of antibody immunity to fungi. For both organisms, the administration of polyclonal antisera to animals has produced mixed results. Thus, immune sera have been protective in some but not all studies (10). Both protective and nonprotective monoclonal antibodies to antigens from *C. albicans* and *C. neoformans* have been identified (10, 75–77). For the latter fungus, there is a close correlation between the molecular composition of the antibody and its biological activity. Protective monoclonal antibodies to the capsular polysaccharide of *C. neoformans* express the V_H family 7183 or T15, whereas nonprotective antibodies do not (11).

Several mechanisms can explain the influential role of antibody (reviewed in reference 10). First, antibodies may act as opsonins that enhance the phagocytosis of fungi. Ingestion of antibody-opsonized fungi may direct the organism into cellular compartments (e.g., phagolysosomes) that are inimical to survival of the fungus. Also, these molecules may promote agglutination of fungal elements, thus constraining the ability of clumped cells to disseminate. Antibodies may bind to circulating fungal substances, such as mannan from *C. albicans* or polysaccharide from the capsule of *C. neoformans*, that modulate the expression of cellular immunity. Antibodies to fungal surfaces may block binding to host cells, which may limit the extent of infection, and they may exert cidal activity or alter morphogenesis (10). Hence, antibodies can exert multiple functions that augment the protective immune response.

T-cell-mediated immunity. Immunocompetent T cells are crucial in host defenses against many pathogenic fungi including *B. dermatitidis*, *C. immitis*, *C. neoformans*, *C. albicans*, *H. capsulatum*, *Paracoccidioides brasiliensis*, and *Pneumocystis carinii*. Numerous studies have demonstrated that animals that lack T cells are more susceptible to infection with these fungi than are those with immunocompetent T cells. Conversely, passive transfer of sensitized T cells can promote elimination of the fungus (3, 5, 6, 9, 18, 38, 46, 48, 65, 69, 70, 96, 98). Both $CD4^+$ and $CD8^+$ T-cell subpopulations have been shown to modify the infection with *H. capsulatum*, *C. neoformans*, *C. albicans*, and *P. carinii* in experimental animals (3, 6, 7, 22, 38, 46, 48, 98). In humans, $CD4^+$ T cells are essential for host defenses, as the experience with AIDS indicates. Most patients who manifest one of these infections often have profound depletion of $CD4^+$ T cells to less than 200/μl.

$CD4^+$ T cells recognize peptide ligands of 13 to 25 amino acids that are bound to the surface of class II major histocompatibility complex (MHC) molecules, whereas $CD8^+$ T cells respond to peptides of 8 to 9 amino acids that are attached to class I MHC molecules (15, 103). The principal mechanism by which $CD4^+$ T cells influence host resistance is by production of cytokines. $CD4^+$ T cells can be separated into two functional categories—T helper 1 (Th1) and T helper 2 (Th2). The former produce IFN-γ and IL-2 and are the primary mediators of host defenses associated with activation of phagocytes (89). In addition, these cytokines contribute to delayed-type hypersensitivity responses (89). Th2 cells, on the other hand, release IL-4, IL-5, IL-10, and IL-13. These molecules are involved in antibody production and promote protective immunity to helminths by stimulating the generation of immunoglobulin E (IgE) and activating eosinophils (89). The polarity of the activities of these cytokines is apparent in some animal models of infection with pathogens such as *Leishmania major*, but both types of T cells may be operative during the evolution of the immune response to an invading microbe (85). Similarly, $CD8^+$ T cells can be classified according to the types of cytokines they release, but much less is understood of the conditions under which they polarize (87).

Another effector mechanism is cytotoxic activity. This function is contained chiefly within the $CD8^+$ T-cell subpopulation, although $CD4^+$ T cells also can express cytolytic activity (73). $CD8^+$ T cells can damage *C. albicans* hyphae and kill *C. neoformans* directly (7, 59, 60, 78). In addition, it is possible that $CD8^+$ or $CD4^+$ T cells can lyse phagocytes or other cell populations harboring fungi, but the relative contribution of this encounter to host defenses is not known. The theoretical scenario is that the cytolytic T cell, through its T-cell receptor, engages a cell infected with fungi and responds to peptides bound to the class I or II MHC on the cell surface, thus causing lysis. Concomitantly, recognition of the peptide-MHC complex by the T-cell receptor prompts the T cell to release macrophage-activating cytokines (e.g., IFN-γ) that can stimulate surrounding phagocytes to express antifungal activity. As a consequence, fungi enter into neighboring phagocytes that are stimulated to restrict growth and not phagocytes that are permissive for replication.

Another primary contribution of T cells is the production of antibody. These cells collaborate with B cells to initiate the synthesis and release of antibodies. T cells that are Th1 promote the synthesis of IgG2a and IgG3, whereas Th2 cells enhance the production of IgM, IgG1, IgA, and IgE (89). Thus, the type of Th cell generated in response to infection or vaccination becomes a key issue since the ability of an antibody to promote fungal elimination may be dependent on the isotype, as has been demonstrated with *C. neoformans*. In experimental cryptococcosis, the hierarchy of efficacy among monoclonal antibodies in fungal clearance is IgG1 > IgG3 > IgM > IgA (75). Thus, both Th1 and Th2-generated antibodies are efficacious in this infectious model although the Th2-associated antibody IgG1 appears to be the most effective.

FUNGAL IMMUNOGENS

If the goal is to create a subunit vaccine composed of inert molecules, one of the most formidable challenges to the production of a useful vaccine is the identification of an immunogen(s) from a fungus. Within a eukaryote, there are potentially hundreds if not thousands of candidate molecules that can stimulate an immune response. Unfortunately, no motifs or canonical sequences exist that distinguish a protective antigen from one that is not. It is conceivable that the protective efficacy of any antigen is dependent on the delivery system. As an example, heat shock protein 65 (hsp 65) from *Mycobacterium tuberculosis* was believed to be a probable vaccine candidate because it induced proliferation in a high proportion of T cells (~20%) from exposed individuals (50). However, no reports were published regarding the utility of this recombinant protein in protecting animals from infection until recently, when Tascon et al. (95) demonstrated that genetic immunization with hsp 65 confers protective immunity in mice. Thus, our concepts of how to determine if a gene or its product can mediate protection must take into consideration the method in which the immunogen is administered in vivo. In the following paragraphs, potentially useful fungal immunogens will be reviewed (Table 1).

B. dermatitidis

B. dermatitidis is endemic primarily to the southeastern and midwestern United States. It infects not only humans but also canines. One protein-containing antigen, WI-1, is found on the surface of *B. dermatitidis* yeast cells (54). It is highly immunogenic and stimulates a humoral and T-cell-mediated response. It also serves as a ligand between phagocyte and fungus and is recognized by the CR3 and CD14 receptors (79). This antigen possesses three functional domains: (i) a central region that contains a 25-amino-acid repeat, arrayed in tandem with 34 copies—the tandem repeat has high homology to invasin from *Yersinia* spp.; (ii) an epidermal growth factor-like region that is present at the C terminus; and (iii) a hydrophobic N-terminal sequence (47, 53). Antibody responses are directed primarily at the 25-amino-acid repeat, and the T-cell determinants are mapped to the amino terminus (53).

Because this protein is immunogenic, its effect on experimental infection has been examined. Immunization of C57BL/6 mice with amounts ranging from 25 to 200 μg produced a

TABLE 1. Catalog of fungal immunogens

Fungus	Immunogen	Protective[a]	Reference(s)
Blastomyces dermatitidis	WI-1	Y	28
Candida albicans	Mannan	Y	44
	Enolase	U	93
	Mannoprotein	Y	66
Coccidioides immitis	C-ASWS	Y	58
	Complement-fixing antigen/chitinase	U	84, 108, 114
	Antigen 2	U	113
	4-HPPD	Y	100
	SOW	Y	99
	33-kDa antigen	U	32
Cryptococcus neoformans	Capsule	Y	77
	Cell wall and cell membrane	U	71
Histoplasma capsulatum	Cell wall and cell membrane	Y	37, 39
	Ribosomal-protein complex	Y	96
Paracoccidioides brasiliensis	gp43	Y	28
Pneumocystis carinii	MSG	Y	98
	p55	Y	92

[a] Y, yes; U, unknown.

delayed-type hypersensitivity response as well as a humoral response. The magnitude of the response was directly correlated with the amount of antigen injected. The humoral response was dominated by the synthesis of IgG1 and IgG2b followed by IgG3 and IgG2a (28). This profile is strongly suggestive of a Th2-like response.

Immunization of mice with 100 μg of WI-1 in Freund's adjuvant conferred a protective immune response to mice challenged with a lethal intranasal inoculum of yeast cells (10^4 cells). Six of eight mice survived for over 30 days, whereas all eight mice given bovine serum albumin in adjuvant died. Histopathologically, the lungs of immunized mice manifested little to no inflammation with few organisms whereas the lungs of controls contained pronounced inflammation and massive numbers of yeasts (28). Thus, WI-1 may mediate protection via a Th2-type response.

C. albicans

C. albicans is a commensal in humans and can exist in two forms—as yeasts or hyphae. *C. albicans* has become a major cause of morbidity among patients infected with human immunodeficiency virus (HIV) and of morbidity and mortality among those in intensive care units or those who have undergone aggressive chemotherapy for malignant diseases or transplantation, especially liver transplantation (35). Both humoral and cell-mediated immunity are involved in host defenses to this pathogen. The challenge for vaccine development is the commensal nature of this organism, since humans are colonized during the early postnatal period. To date, no single protein has been shown to mediate protection; one candidate protein is enolase. This protein is recognized by lymphocytes from *C. albicans*-exposed animals, and it induces a humoral response (93). However, its protective efficacy has not been determined.

Surface mannans of *C. albicans* function as adhesins during the attachment of yeast cells to murine splenic macrophages (44). This fraction has been packaged in liposomes and used to vaccinate BALB/c mice. The mannans induced a protective immune response in a model of disseminated candidiasis, and the ability to induce resistance was associated with the presence of circulating agglutinins to the mannan fraction. Antiserum from vaccinated mice could passively transfer protection as well as a monoclonal antibody to the mannan fraction (44). Thus, the elicitation of antibodies to mannan provides a protective mechanism.

A mannoprotein fraction (MP-F2) from the cell wall of *C. albicans* yeasts is immunogenic in mice. This fraction preferentially stimulates $CD4^+$ T cells to produce IFN-γ but not IL-4 (66). Moreover, immunization of mice with MP-F2 conferred a modest reduction in candidal CFU and prolongation of mean survival time compared to controls (66). The molecule(s) within this extract that are responsible for the protective efficacy has not been identified.

C. immitis

Several potential immunogens have been identified from *C. immitis*. The soluble conidial cell wall fraction (SCWF) from *C. immitis* is immunoreactive with T cells from mice exposed to this fungus (52). By using a polyclonal antiserum to this antigenic extract, one antigen subsequently was identified by immunoscreening a *C. immitis* cDNA library. The fusion protein not only recognized antibody but also stimulated T cells from exposed mice. Cloning and sequencing of the gene encoding this antigen revealed it to be the enzyme 4-hydroxyphenylpyruvate dioxygenase, which converts the 4-hydroxyphenylpyruvate to homogenizate (107). Recombinant protein was used to immunize BALB/c mice that were subsequently challenged with *C. immitis* arthroconidia. At 14 days of infection following intraperitoneal challenge, there was a 1 $\log_{10}$ unit reduction in the fungal burden in the lungs of immunized mice compared to controls (100). Subsequently, the same group of investigators has tested the immunogenicity of the extract from the spherule outer wall (SOW). BALB/c mice were immunized with SOW and challenged with arthroconidia intraperitoneally. Lungs from immunized mice contained a three- to fourfold reduction in CFU compared to infected controls (99). Within the SOW, two proteins of 58 and 66 kDa stimulated T cells. These two proteins, which have not yet been identified, also are potential vaccine candidates.

An alkali-soluble, water-soluble antigen (C-ASWS) from *C. immitis* mycelia has successfully been used to vaccinate mice. Injection of 1 mg of this antigen suspended in Freund's adjuvant protected DBA/2J mice, a strain of mouse relatively resistant to *C. immitis* (51), against an intranasal challenge with 50 or 500 arthroconidia (58). There was a significant survival advantage of immunized mice, but the antigen was not completely protective. Furthermore, a challenge of 1,500 arthroconidia overwhelmed the beneficial effect of C-ASWS. These results indicate that, on occasion, the effect of a vaccine may be mitigated by the size of the inoculum. The impact of these studies would have been enhanced if the efficacy of C-ASWS could have been examined in a more susceptible strain of mouse such as BALB/c (51).

A 33-kDa antigen has been isolated from the wall of mature spherules. This antigen is recognized by sera from humans who have recovered from infection and subjects who were vaccinated with killed spherules (36). It also stimulates human T

cells (36). The gene encoding this antigen has been cloned and sequenced. The protein contains a 4-amino-acid repeat, Thr-Ala-Glu-Pro, from amino acids 98 to 141 (32). The identity of this protein is not known since there are no homologous genes or proteins in GenBank. Since this protein is found in spherules which are protective in mice, it is conceivable that the 33-kDa protein can mediate protection.

Additional immunogens from *C. immitis* have been cloned and sequenced. These include antigen 2 (113) and the complement fixation/chitinase gene (84, 108, 114). Although these are antigenic, their influence on host resistance remains to be determined.

C. neoformans

There has been much progress in the development of a vaccine for *C. neoformans* infection. The discovery that monoclonal antibodies to the capsular polysaccharide could mediate protection has led to rapid advances in the field. The capsular polysaccharide, which is innately poorly immunogenic, has been conjugated to tetanus toxoid. Injection of mice with this immunogen stimulates the production of antibodies that possess the same specificity as antibodies generated during active infection. Both sources of antibodies bind to the same antigenic determinant on glucoronoxylmannan (12). Moreover, the vaccine can elicit protective antibodies, and their specificity is similar, if not identical, to that of antibodies found in active infection (26, 27). This finding suggests that the conjugate may be useful for vaccination. In fact, a phase I trial has been under way to determine the safety and efficacy of this vaccine in human subjects (106). In two groups of normal healthy subjects, injection of 25 or 50 μg of the conjugate produced IgG titers that were 38- to 70-fold higher than in preimmune serum, respectively. Adverse reactions were negligible. HIV-infected subjects also were injected without serious side effects (106). Thus, this vaccine is antigenic and appears in this small study to be safe.

One mechanism by which antibodies to the tetanus toxoid-glucuronoxylomannan conjugate may promote protective immunity is by mediating opsonization. However, immunization with this conjugate does not uniformly induce opsonic antibodies in all healthy individuals. In a recent study, one of nine subjects immunized with tetanus toxoid-glucuronoxylomannan failed to generate an opsonic antibody response (112). This finding may be a problem if opsonization is the primary mechanism by which antibody prevents infection. Also, this report raises the caveat that the vaccine may not function optimally in immunodeficient individuals. This problem is highlighted by the demonstration that sera from HIV-infected subjects who received the vaccine contained different specificities for peptide mimotopes from those of uninfected individuals (110).

Proteins in the cell wall and cell membrane of this fungus are stimulatory for lymphocytes from adult humans and fetal cord blood. Thus, a mitogen or superantigen may be present in this fraction of *C. neoformans* (71). Additional data will have to be forthcoming to determine if a component of this extract can be protective. One of the major impediments in the search for immunogenic proteins from *C. neoformans* is the presence of a high degree of glycosylation, which limits the detection of specific proteins. Thus, highly glycosylated proteins often cannot be identified by gel electrophoresis because of the amount of carbohydrate that is present. The carbohydrate is observed as a smear, and proteins are not apparent. Once this problem can be overcome, it is anticipated that one or more protective proteins will be identified.

H. capsulatum

Two early reports identified protective immunogens from *H. capsulatum*. One was an ethylenediamine extract from the cell wall and cell membrane (CW/M) of this fungus, and the other was the ribosomal-protein complex (37, 96). Unfortunately, additional mapping of the immunogenic determinants was not reported.

We initiated a series of studies to identify protective immunogens from *H. capsulatum* several years ago. A detergent extract from the CW/M of the yeast phase was prepared and analyzed for its capacity to induce a protective immune response when injected with adjuvant into mice (39). Once it had been demonstrated that the CW/M could confer protection, the technique of T-cell Western blotting (39) in conjunction with murine T-cell clones was used to ascertain if the responses by T cells could be isolated to one or a few proteins (39). T cells reacted to a region of the T-cell Western blot that spanned a region of 56 to 80 kDa of the CW/M. Based on these findings, one protein, termed HIS-62, was isolated by electroelution from preparative sodium dodecyl sulfate-polyacrylamide gels (41). That protein was subsequently shown to stimulate cellular immune responses in mice exposed to *H. capsulatum*. In addition, immunization with 160 μg of protein conferred protection against a lethal intravenous challenge in three strains of mice, BALB/c, C57BL/6, and CBA/J.

We identified HIS-62 as hsp 60 by cloning and sequencing the gene encoding this protective immunogen (40). Recombinant hsp 60 was produced in a prokaryotic expression system and tested for the ability to protect BALB/c mice from a lethal intranasal challenge of *H. capsulatum* yeasts. Injection of 400 μg of recombinant hsp 60 protected 100% of mice, whereas 89% of infected controls died over a 45-day period. Thus, hsp 60 from this fungus can immunize mice against either an intravenous or an intranasal challenge.

Subsequently, we generated polypeptides from hsp 60 to determine if the protective efficacy would map to a specific region of the protein. Four overlapping cDNA fragments were amplified by the PCR and cloned into the expression plasmid, pET19b. The four fragments were tested for their capacity to stimulate lymphocytes from mice immunized with *H. capsulatum* or recombinant hsp 60 and to confer protection against sublethal and lethal intranasal challenges. All of the expressed polypeptides stimulated lymphocytes from BALB/c mice immunized with yeasts and with recombinant hsp 60. Fragment 3 induced the most vigorous response by cells from yeast-immunized mice, whereas fragment 1 caused the greatest stimulation by cells from hsp 60-immunized animals. Splenocytes from yeast-immunized C57BL/6 mice did not react with any fragment; in contrast, cells from mice immunized with hsp 60 responded to each fragment, although fragment 2 caused the most intense stimulation. In both strains of mice, fragment 3 conferred protection against sublethal and lethal challenges (24). Thus, a common protective domain of hsp 60 has been identified. Furthermore, there is no correlation between in vitro reactivity and the ability to mediate protection.

A second protective immunogen from *H. capsulatum* has been isolated and called HIS-80 because its molecular mass is 80 kDa. This protein also was isolated from CW/M, and it has homology at the amino terminus to the hsp 70 family (42). This protein stimulated the proliferation of splenocytes from C57BL/6 mice immunized with yeasts or with HIS-80, and immunization with it produced a ~60% reduction in CFU in spleens of mice challenged intravenously with a sublethal inoculum (42).

Since HIS-80 did manifest homology to the hsp 70 family, we

cloned, sequenced, and expressed the hsp 70 gene from *H. capsulatum* and tested it for protective efficacy. The hsp 70 gene was expressed in pET19b and used to immunize C57BL/6 mice. Mice given the recombinant hsp 70 succumbed to a lethal intranasal challenge in a time course similar to that of controls (2). Immunization did not produce a protective response in mice given a sublethal challenge. Thus, HIS-80, although manifesting homology to hsp 70, is not hsp 70. Based on molecular weight and the amino acid sequence of the amino terminus, this protein is most probably a member of the BiP family (2). Attempts to isolate the gene encoding this antigen have not been successful.

P. brasiliensis

Paracoccidioidomycosis is a fungal disease that is characterized by a pyogranulomatous tissue reaction. It is found principally in the tropical and subtropical areas of South America. As with other fungal pathogens, protective immunity has been correlated with the presence of an exuberant cell-mediated immune response. No protective immunogen has yet been reported, but the major diagnostic antigen, a glycoprotein with a molecular mass of 43 kDa (gp43), has shown promise as a potential protective antigen. This antigen has been cloned and sequenced and has homology to exo-β-1,3-D-glucanase from *Saccharomyces cerevisiae* and *C. albicans* (16). Functionally, it acts as an adhesin since it binds to laminin-1 (104). gp43 elicits an immunodominant humoral response as well as a cellular immune response (101). $CD4^+$ T cells preferentially react to gp 43 in vitro, and they synthesize IFN-γ in response to it. This cytokine is critically important in controlling infection in mice (28). Therefore, it is likely that the production of IFN-γ following injection of gp43 may promote fungal clearance. Preliminary data indicate that immunization can reduce the burden of *P. brasiliensis* in the lungs of mice inoculated intratracheally and reduces the severity of inflammation in lungs (28).

P. carinii

A major surface antigen of *P. carinii*, known as major surface glycoprotein (MSG) or glycoprotein A, is an immunodominant antigen that is recognized by sera from animals or humans exposed to this fungus and subserves as an adhesin function (80). Spleen cells and $CD4^+$ T cells from rats naturally exposed to *P. carinii* were stimulated in vitro with native MSG and transferred to corticosteroid-treated Lewis rats with pneumocystosis. The organism burden was significantly reduced compared to that in infected controls. However, when only $CD4^+$ T cells were used, some animals developed a hyperinflammatory response that led to their premature demise although these animals contained fewer organisms (98).

Additional studies have been conducted on the influence of immunization with native MSG on the course of pneumocystosis in corticosteroid-treated rats. Injection of MSG into rats prior to administration of corticosteroids leads to a reduction in the burden of *P. carinii* compared to that in controls (97). Similarly, immunization with recombinant p55, a protein derived from rat pneumocystis without a defined function, also protects corticosteroid-treated rats (91). The mechanisms whereby these antigens promote the protective immune response remain unknown, although it is likely that humoral immunity is dominant since T-cell function in steroid-treated animals is greatly depressed. Thus, these two antigens provide a promising beginning in the search for a vaccine for this opportunistic pathogen.

Adhesins as Protective Immunogens

The aforementioned findings concerning the search for protective immunogens from fungal pathogens raise an interesting issue concerning the protective efficacy of molecules that act as ligands to cell surfaces. Mannan, WI-1, gp43, and MSG function as adhesins, and each can mediate protective immunity presumably by stimulating production of antibodies, T cells, or both. The ability of these antigens to mediate both functions may be serendipitous; alternatively, it raises the possibility that fungal adhesins are an excellent target to pursue as putative vaccine candidates. Support for this contention will have to await other reports that adhesins from several fungi are protective.

APPROACHES TO VACCINATION

Conventional Methods

The most common approach to experimental fungal vaccination at this time has been to identify inert fungal substances such as proteins or carbohydrates for vaccination. These types of components are the safest since they do not involve the use of replicating microbes whose virulence may be altered. However, native protein or carbohydrate purification has limitations because of yield and purity concerns; therefore, many investigators have employed recombinant technology to generate large quantities of a protein-containing immunogen. Expression has been performed principally in prokaryotic systems because of ease of use and the quantity of recombinant protein generated. However, ongoing work has involved *S. cerevisiae*, *Pichia pastoris*, and baculovirus. Thus, hsp 60 from *H. capsulatum* (40), MSG and p55 from *P. carinii* (91, 97), WI-1 from *B. dermatitidis* (28), and 4-hydroxyphenylpyruvate dioxygenase from *C. immitis* (107) represent several examples of recombinant fungal proteins that are expressed by prokaryotes and that are undergoing examination of their immunobiological function.

Recombinant technology also permits fine mapping of the immunogenic determinants of a protein. The identification of the smallest fragment that can mediate protection is important, since irrelevant or harmful moieties of the protein can be eliminated. One problem that may be anticipated with production of peptides or small protein fragments is that they may not be able to be expressed by prokaryotic expression systems because of size. Another potential problem is that a peptide may be restricted by a single MHC allele. Therefore, in large populations of humans, the efficacy may be limited if that MHC allele is lacking in a high proportion of individuals. Despite these caveats, peptides offer promise as immunogens because of their simplicity.

Immunogenic carbohydrates such as glucuronoxylomannan from the capsule of *C. neoformans* or mannans from *C. albicans* still require conventional biochemical purification for isolation. No large-scale organic synthesis of such molecules is yet possible. This fact may be a major impediment to production of these antigens for widespread use. Carbohydrates are weakly immunogenic, and they are useful only for eliciting a humoral response. Furthermore, they often require a carrier to make them potent. Cryptococcal glucuronoxylomannan has been chemically conjugated to tetanus toxoid (12). This linkage converts the polysaccharide from a T-cell-independent to a T-cell-dependent antigen, thus enhancing substantially the immunogenicity of the polysaccharide. Mannans from *C. albicans* have been incorporated into liposomes to create an immunogenic substance (44).

Adjuvants

Soluble proteins when delivered intradermally or intramuscularly require an adjuvant. The selection of an adjuvant becomes a critical issue in the endeavor to generate an appropriate immune response for each fungus. Aluminum salts (alum) were one of the first adjuvants to be used, and they remain the only one for human use. Their efficacy is limited largely to promoting antibody responses to immunogens (43). In experimental animals, Freund's adjuvant has been most frequently used. However, it is clearly too toxic for human use. Newer agents include immune stimulating complexes (49, 105), adjuvant peptides, and bacterial components. Galactose oxidation of T-cell surfaces has been shown to enhance immunogenicity. Thus, exposure of murine lymphocytes and antigen-presenting cells to galactose oxidase increases the number of amine-reactive carbonyl groups, which enhances the interaction between these two types of cells (111). More recently, it has been reported that complement component C3d augments the antigenicity of hen egg lysozyme by molecular modification of this protein (25). Thus, a constituent of the innate immune system can be used to enhance the antigenicity of proteins. It remains to be determined if this molecule will promote the protective efficacy of immunogens. Most investigators have used conventional adjuvants with fungal immunogens such as Freund's, monophosphoryl lipid A plus streptococcal cell wall skeleton, or other oil-based adjuvants. None have been found to be superior.

Many adjuvants often elicit a predominantly $CD4^+$ or Th cell response. In fungi, this subpopulation of cells appears to be the central T-cell family that confers protection either through effector function or by collaborating with B cells to stimulate humoral immunity. However, should a dominant $CD8^+$ T-cell response for one or more fungi be critical, immune stimulating complexes or detergent-based adjuvants (e.g., Quil-A) will direct the immune response to an antigen into this pathway (49, 62, 72, 105). Thus, the subset of T cells that needs to be activated to confer protection is influenced by the adjuvant.

Cytokines as Adjuvants

Cytokines are an exciting new addition to the armamentarium of adjuvants. As an example, the monokine IL-12 can act as an adjuvant. Injection of a crude antigenic extract from *L. major* in the absence of IL-12 does not induce a protective immune response against this parasite in mice, whereas concomitant administration of IL-12 with the extract substantially reduces the burden of infection (1). The mechanism of action appears to be that IL-12 enhances the Th1 response and, as a consequence, production of IFN-γ, which is key in the generation of a protective immune response in mice to this pathogen. Although this cytokine exhibits great promise, we have investigated its role in vaccination with the H antigen from *H. capsulatum* (23). This antigen induces a cellular immune response in mice, but it does not mediate protection in mice. Therefore, we sought to determine if coadministration of H antigen with IL-12 to mice would alter the protective efficacy of this antigen. Either this cytokine was given intraperitoneally at the time of injection of H antigen, or, in a second experiment, it was admixed with H antigen. H antigen alone or with IL-12 was given twice to animals. The injections were separated by 2 weeks. In neither case did the injection of IL-12 convert H antigen into a protective immunogen (21), although *H. capsulatum*, like *L. major*, is dependent on a Th1 response. Experiments to generate a fusion protein with IL-12 and H antigen, to determine if this approach would create a protective immunogen, are planned.

Similarly, a fusion protein consisting of anti-B-cell lymphoma idiotype and GM-CSF has created a potent immunogen that can protect animals from a challenge with the lymphoma line 38C13 (94). This development in tumor immunity may be transferable to infectious diseases including fungal diseases.

DNA Vaccination

Conventional methodologies of vaccination are giving way to newer approaches, of which DNA vaccination represents one of the most exciting, not only for fungal diseases but also for all diseases mediated by pathogenic microbes. The primary method at present is to use plasmid-encoded DNA that does not replicate and inject it into muscle with or without an irritant such as cardiotoxin (31, 95, 102). Bombardment into the skin of DNA bound to particles is another delivery system that is available for immunization. One of the recent developments noted with DNA delivery by a gene gun is that a Th2 response is dominant whereas DNA delivered by intramuscular injection produces a Th1 response (34). Thus, for pathogenic fungi that require a Th1 response for clearance, vaccination with a gene gun may not be suitable.

In several systems, DNA vaccination has induced both cellular and humoral immune responses and has generated a protective immune response. One of the most interesting features is that injection of DNA encoding the hsp 65 gene from *M. tuberculosis* has resulted in a protective immune response whereas injection of the protein emulsified in adjuvant has not (95). The reason for the success of the genetic vaccine is that it induces a vigorous cytotoxic T-cell response that is necessary for the elimination of *M. tuberculosis* (95). This finding raises a whole series of questions regarding screening for vaccine candidates. One issue is that the lack of efficacy of a protein as an immunogen is no longer suitable for excluding it as a vaccine candidate. Indeed, it may function more effectively if used as a genetic vaccine. However, more evidence needs to be accumulated to determine whether the data with hsp 65 from *M. tuberculosis* can be extended to genes from other pathogens.

One of the attractive features of genetic vaccination is that the genes often are expressed within the endogenous pathway of antigen processing, which will lead to cytolytic T-cell activation (109). The arming of this T-cell subpopulation may be desirable for a number of pathogens, including fungi, in which $CD8^+$ T cells play a prominent role in host defenses. Thus, if one goal of vaccination is to elicit protective $CD8^+$ as well as $CD4^+$ T cells, genetic vaccination is superior to the use of conventional proteins which will enter the exogenous pathway, thus leading to activation of $CD4^+$ T cells almost exclusively.

Barry et al. (4) have reported a novel method for screening putative vaccine candidates by using digested DNA of *Mycoplasma pulmonis* to vaccinate mice. This approach would supplant the problems of using a live vector. An expression library was constructed in three frames and ligated to the gene encoding human growth hormone. DNA was injected into mice that were subsequently challenged with *M. pulmonis*. The CFUs of vaccinated mice were considerably lower than those of controls. This approach has several advantages in that it does not require a priori knowledge of the antigen and it eliminates the risk of a live vaccine. However, the genome of *M. pulmonis* is small ($\sim 10^6$ bp) and thus is readily manipulated. The use of a larger genome, such as is found in the pathogenic fungi, might not be as fruitful as with pathogens that possess smaller genomes. The more complex the genome, the more likely one is to discover antigens that suppress immune responses rather than promote them.

A third method that has been reported is to transfect dendritic cells, which are potent antigen-presenting cells, with naked DNA and assess the impact on protective immunity (17). This approach is clearly feasible for pathogenic fungi.

DNA vaccination holds promise in the field of mycology. At present, however, no laboratory has reported the utility of DNA vaccination in a medically important fungal infection. However, the results with *M. tuberculosis* suggest that a nonprotective protein may indeed become protective when used in an alternative manner. One avenue of investigation that should be pursued is to determine the mechanisms by which an immunogen that is delivered by DNA vaccination is active.

Altered Fungus

Most successful viral vaccines are based on the isolation of an attenuated strain. Hypovirulent or avirulent strains of fungi have been described for several species, but it is not known whether they are protective against many strains that exist in nature. The use as a vaccine of a fungal strain with diminished if not absent virulence certainly has potential, but several risks are involved. First, the traits that make the fungus less virulent would have to be stable. In addition, for the deep mycoses, the vaccine fungus would have to be eliminated by the host since it otherwise could form a nidus for reactivation. Genes that encode the replication cycle would have to be deleted or damaged by molecular manipulation. An attractive possibility for the deep fungi is the use of the saprophytic form. As an example, treatment of the mycelial phase of *H. capsulatum* with the sulfhydryl blocking agent, *p*-chloromercuryphenylsulfonic acid, inhibits the ability of fungal elements to convert to the yeast phase (64). These crippled fungal elements, when injected into mice, protect the animals from a challenge with a lethal inoculum of yeast cells (64). Thus, it may be possible to incapacitate important components of the fungal cell cycle that allow propagation yet maintain immunogenicity.

Therapeutic Vaccination

The use of an immunogen to treat an infectious disease rather than just to prevent it has emerged as an addition to the potential use of vaccine candidates. This therapeutic option has arisen principally in the area of viral infections that either reactivate (e.g., herpes simplex virus) or mutate in vivo with a high frequency (e.g., HIV) (92, 105). Although much of this work is in the nascent state, the currently licensed rabies vaccine is used therapeutically in individuals who have been exposed to the virus. The most likely mechanism to account for the activity of the immunogen is that it boosts antigen-specific immunity during active infection. This enhancement would lead to a clearance of the invading pathogen. Because a high proportion of the fungal diseases are thought to involve reactivation of dormant foci, therapeutic immunization becomes a compelling approach to eliminate dormant organisms or to heal active infection, especially in life-threatening disease.

VACCINATION OF THE IMMUNOCOMPROMISED HOST

One of the major controversies and challenges surrounding fungal vaccination is the case of the immunocompromised host. As mentioned in Introduction, many of the fungal infections develop in a patient whose immune system has been altered by pharmaceutical agents or by HIV infection. It is clear that vaccines that rely on a competent immune system to function may not be efficacious in these patients. Certainly, vaccines that elicit protective antibodies may be more useful in these conditions than in those that strictly rely on cellular immunity, because of the longevity of circulating antibodies. However, even antibody-centered vaccines would eventually lose efficacy in patients whose immune systems are compromised over months to years. The challenge will be to enhance the effect of a vaccine when the immune system is dysfunctional. One approach will be to link the vaccine with attempts to restore the integrity of the immune system. The delivery of a vaccine in combination with cytokine or cytokines that are known to enhance the immune system would be one approach. Another would be to link the administration of the vaccine with infusion of immunocompetent T cells or B cells to promote the immunogenicity of the vaccine. Obviously, a thorough understanding of the mechanisms of protection will be necessary before any of these concepts can be tested. Moreover, it is imperative that many vaccine candidates be tested in immunosuppressed animal models.

Another dilemma that must be confronted is that clinicians do not yet have the means to identify those immunocompromised individuals who will develop fungal infections. Although risk factors are known, not every individual at risk manifests a serious fungal infection. As an example, Cincinnati is an area where *H. capsulatum* is highly endemic, and it would be anticipated that nearly all HIV-infected patients manifest disseminated histoplasmosis once their $CD4^+$ T-cell counts become less than 200/μl. However, only 12 to 15 new cases are detected per year, although the number of patients at risk is 10 to 30 times greater than the number who manifest the infection. Therefore, newer methods are necessary to identify who is at highest risk in order to determine who would benefit the most from vaccination or a combination of vaccination and immunorestorative therapy.

CONCLUSIONS

The concept that fungal vaccines could be useful is no longer merely an illusion. There is a need for either preventive or therapeutic vaccines to curb the rising incidence of fungal infections. Although the knowledge base is incomplete, a great deal of progress has been made to understand the basic mechanisms of host defense. This information is fundamental to the creation of useful vaccines. Early successes in animal models must be translated into treatment for humans. The search for immunoreactive molecules is difficult, and the initial discoveries may not be sufficient. However, they provide a strong framework with which to continue the discovery process.

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