The Second NIAID Workshop in Medical Mycology: Molecular and Immunologic Approaches to the Diagnosis and Treatment of Systemic Mycoses

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PREFACE

The NIAID recognizes medical mycology as an area in need of development. An Institute-sponsored workshop on "Mycology Research in the 1990's" in Chicago, Illinois, 28-29 September 1991 addressed the increasing importance of medical mycology. Twenty medical mycologists from throughout the United States were invited to discuss the issues and to conceptualize and condense the active research areas into topic areas in need of development.

Five areas were targeted for focus. These were molecular mycology, diagnosis and treatment, immunology, antigen structure and function, and epidemiology. Each of these five topic areas was targeted for development into a separate workshop/minisymposium, co-sponsored by the NIAID and educational grants raised by the medical mycological community.

"Molecular Medical Mycology," the first workshop in the series, was held in Minneapolis, MN on 24-26 June, 1993 and chaired by Dr. Paul T. Magee. One hundred and forty-seven mycologists attended and exchanged ideas. A key to the success of information exchange was the utilization of "break out" sessions that provided an informal setting for free exchange of ideas, an opportunity for a more active involvement for all of the participants, and an environment fostering new collaborations.

"Molecular and Immunologic Approaches to the Diagnosis and Treatment of Systemic Mycoses," the second workshop in the series, was held on the campus of Northern Arizona University, Flagstaff, Arizona, 8-11 June 1994. The workshop format was modeled after the first in the series, and was attended by 80 registrants.

I believe the workshop series continues to be successful in accomplishing the stated goals, and that this success is representative of the field of medical mycology whose time has come. Finally, I would like to thank all those who contributed to the success of this workshop, including all of the participants, organizing and writing committees, and especially Drs. John algiani and Michael Pfaller who chaired and took lead roles in organizing this outstanding event whose findings are still timely.

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INTRODUCTION

The workshop was designed to present an overview of the present status of the diagnosis and treatment of systemic mycoses and to encourage innovative molecular and immunologic

approaches directed at the public health needs. An opening theme lecture summarized the available diagnostic and therapeutic strategies and gave an indication of perceived needs. Five sessions presented representative research approaches in the field and several presentations highlighted parallel developments in related fields. A panel discussed examples of partnerships involving industry, the CDC, FDA, and NIH. "Break-out" sessions of 11 participants each were led in discussion by facilitators who summarized the results in separate "at large" sessions.

Key Concepts

- Better diagnostic tests are badly needed for invasive candidiasis and aspergillosis.
- Priorities for therapeutic advances include aspergillosis, candidiasis, and a series of opportunistic mycoses that have been inadequately studied (infections caused by Zygomycetes, dematiaceous fungi, *Fusarium*, etc.).
- Better incidence data are needed to determine the magnitude of the problems, to help establish priorities, and to interest alternative funding sources such as industry.
- More basic research on the medically important fungi is essential and should include not only molecular biology but also physiology.
- Medical mycology can offer model systems for the opportunistic infections that are expected to increase in the 21st century.
- A better understanding of the mechanisms and incidence of drug resistance is imperative, as it appears the medically important fungi will be no different than any other microbes in entering the post antibiotic era.
- A Fungal Genome Center should be established for one or more model fungal pathogens (e.g., *Candida albicans* and *Aspergillus fumigatus*) with support sought from multiple sources.
- Vaccine research should be applied to the medically important fungi.

STATUS AND LIMITATIONS OF DIAGNOSIS AND THERAPY OF SYSTEMIC FUNGAL INFECTIONS

Current Status

Culture of body fluids or tissue for fungus continues to be the mainstay of diagnosis of systemic fungal disease. Unfortunately, definitive identification of a fungus by culture may take one to four weeks. A presumptive diagnosis can be made on the basis of characteristic histopathology and special tissue stains such as periodic acid-Schiff (PAS) and Gomori-methenamine silver (GMS). Skin tests with antigens such as histoplasmin or spherulin, while useful in epidemiologic studies, have no role in diagnosis of active disease. Serologic tests have limited values for most fungal diseases because of low specificity and sensitivities; however, there are exceptions which include the latex agglutination test for cryptococcal antigen in patients with suspected cryptococcosis, and the complement-fixation and immunodiffusion tests for coccidioidomycosis and paracoccidioidomycosis. At present, highly specific and sensitive serologic tests are not available for early and rapid diagnosis of candidiasis and aspergillosis, the two most common and clinically important opportunistic fungal diseases in immunocompromised hosts. Investi-gational approaches to diagnosis and monitoring of systemic mycoses include: (1) detection of unique fungal metabolites by gas liquid chromatography or antigen/antibody based methods; (2) detection of immunodominant fungal antigens by radioimmunoassay, ELISA, latex agglutination or immunoblot; and 3) use of molecular probes such as polymerase chain reaction to detect fungal DNA. While results of some newer tests to identify *Candida* cell wall mannan, *Candida* cytoplasmic enolase antigen, and *Aspergillus* galactomannan are promising, drawbacks persist. For example, these tests often must be performed on multiple sera over time in order to achieve

acceptable sensitivity, and have restricted availability because they are not commercially produced, and have low perceived market priority.

At present, the five licensed antifungal drugs which are the most important and commonly used agents against the systemic fungal diseases are amphotericin B, flucytosine, and the three azole drugs, ketoconazole, itraconazole, and fluconazole. Ergosterol, the principal sterol in the fungal cytoplasmic membrane, is the target site of action of amphotericin B and the azoles. Amphotericin B, a polyene, binds irreversibly to ergosterol, resulting in disruption of membrane integrity and ultimately cell death. The azole drugs inhibit synthesis of ergosterol through an interaction with the cytochrome P450 dependent enzyme, 14 alpha demethylase, necessary for the conversion of lanosterol to ergosterol. By contrast, flucytosine, an oral fluorinated pyrimidine, inhibits both fungal DNA and RNA protein synthesis. While these drugs are effective therapies for many of the systemic mycoses including blastomycosis, candidiasis, cryptococcosis, histoplasmosis, and sporotrichosis, they are only minimally or moderately effective for aspergillosis, coccidioidomycosis, non-albicans candidiasis, and phaeohyphomycosis caused by dematiaceous moulds. Moreover, these drugs are not without other problems. Amphotericin B, considered by some authorities as the "gold standard" because of its broad spectrum of activity and fungicidal action, is available only as an intravenous formulation in the United States, requiring continuous intravenous access, and is associated with significant dose-limiting toxicity, especially nephrotoxicity. Flucytosine also has the potential for considerable toxicity, especially affecting the bone marrow, liver, gastrointestinal tract, and skin. As a result, this drug has never been optimally utilized. Although the azole class of drugs is an important advance and offers effective and safe alternatives to amphotericin B and flucytosine for many of the systemic mycoses, the azoles, too, are not ideal drugs. Only fluconazole is available as both an oral and intravenous formulation. Although amphotericin B and flucytosine, the azoles are potentially hepatotoxic, may inhibit steroid hormone synthesis in humans, and have the potential to interact with many classes of drugs (oral hypoglycemics, oral anticoagulants, phenytoin, cyclosporine, H1 and H2 receptor antagonists, rifampin, etc.) with serious sequelae. In addition, fluconazole-resistant *Candida* species are an increasing cause of concern in AIDS patients and other compromised hosts exposed to fluconazole and as a prophylaxis over extended periods.

Because of the toxicity and other problems associated with the currently available antifungal drugs, new treatment approaches are needed. Current efforts in antifungal drug development are focused on: (1) drugs with novel fungal cell targets; (2) investigational azole and allylamine drugs; and (3) novel formulations of currently licensed drugs. In addition, recombinant cytokines, both licensed and investigational, have promise. With regard to novel fungal cell targets, an important difference between fungi and mammalian cells is that the fungal cell envelope consists of a cell wall, composed primarily of two carbohydrates, glycan and chitin, and mannoproteins, and cytoplasmic membrane. By contrast, cell walls are not found in mammalian cells and other eukaryotic cells. Available evidence indicates that drugs which target fungal cell wall structures will not adversely affect mammalian cells; thus, the potential for toxicity of such drugs in humans is reduced. Examples of investigational drugs which interrupt formation of the unique fungal cell wall include: (a) nikkomycins which inhibit chitin synthase; (b) the echinocandin/pneumocandin/ lipopeptide class which inhibit glycan synthesis; and (c) pradimicins which exhibit calcium dependent binding to mannan (mannoprotein). The investigational azole drugs under development by several pharmaceutical companies have the potential to outperform the currently licensed azoles, ketoconazole, itraconazole, and fluconazole, in terms of broader spectrum of activity, increased efficacy, and less toxicity. The allylamines, a new class of drug, inhibit squalene epoxidase, another enzyme in the biosynthetic pathway of ergosterol.

Novel formulations of currently licensed drugs are aimed at enhancing the efficacy and reducing the toxicity of the parent compound. There are two classes of drugs in this group. The first class are lipid formulations of amphotericin B including liposomal amphotericin B (AmBisome), amphotericin B colloidal dispersion (Amphocil), and amphotericin B lipid complex (ABLC). Enveloping amphotericin B in liposomes or complexing the drug with lipids offers several

advantages of the novel formulations over conventional amphotericin B, which is complexed in desoxycholate, a bile salt. These potential advantages include: (a) up to 5- 10-fold increase in daily dose of amphotericin B, thereby increasing the therapeutic index; (b) less nephrotoxicity, the major limiting toxic effect of conventional amphotericin B; (c) tropism for reticulo-endothelial organs such as lymph nodes, liver and spleen where fungi typically home in contrast to the tropism of conventional amphotericin B for kidneys; (d) preferential binding of lipid formulated drugs to fungal cell membranes; and (e) efficacy in some patients who have failed or cannot tolerate conventional amphotericin B.

New oral and intravenous preparations of itraconazole represent a second class of novel formulations. Itraconazole, currently available only for oral administration, is a weak base which requires an acid environment for optimal solubilization and absorption. The bioavailability of itraconazole is increased when it is formulated in hydroxypropyl- beta-cyclodextrin, a cyclic oligosaccharide carrier molecule that increases the solubility of lipophilic compounds in aqueous solutions. Hydroxypropyl-beta- cyclodextrin itraconazole may be especially helpful in selected clinical situations, such as AIDS patients with achlorhydria or receiving concurrent drugs which impair absorption, or critically ill hospitalized patients who cannot take oral medications.

Immunomodulators may be important adjuncts to therapy of systemic mycoses. Invasive fungal diseases are increasingly observed in immunocompromised patients, especially those with protracted granulocytopenia secondary to neoplastic disease or cytotoxic chemotherapy, transplant recipients receiving immunosuppressive drugs such as high dose corticosteroids and cyclosporine, and AIDS patients with progressively declining CD4+ T cells and other perturbations in immune function. Current evidence indicates that both granulocytes, adequate in number and function, as well as intact cell-mediated immunity, are keys to successful outcome in patients with opportunistic yeast and mould diseases; cellular immunity also plays an important role in the host's containment of the endemic mycoses. Recent *in vitro* and animal *in vivo* data suggest a role in the management of fungal diseases for the expanding array of recombinant cytokines, especially interferon-gamma, the colony- stimulating factors, the various interleukins or interleukin antagonists, as well as passive immunotherapy with monoclonal antibodies. To date, only limited trials with these agents have been performed in humans with fungal diseases.

Recommendations

- Encourage continued investigation of novel approaches to serodiagnosis of systemic mycoses, especially candidiasis and aspergillosis. Evaluation of new diagnostic methodologies should be incorporated into large scale prospective clinical trials.
- Continue to develop novel antifungal drugs, i.e., new classes of drugs. Pursue new fungal cell targets and new formulations of existing drugs.
- Give priority to drug development programs which focus on aspergillosis and diseases caused by other moulds, among the most common emerging opportunistic mycoses.
- Explore more aggressively the role of immunomodulators in the management of systemic fungal diseases. Combinations of immunomodulators plus antifungal drugs should be evaluated in Phase II and III clinical trials.

TARGETS FOR INHIBITING PROLIFERATION OR VIRULENCE

Current Status

A major concern in treatment of fungal infections is the limited number of efficacious antifungal drugs. This problem is being compounded by the rapid emergence of resistant organisms which further diminishes therapeutic capabilities. The discovery and development of new antifungals could be greatly facilitated by a fundamental knowledge of cell proliferation as it relates to pathogenic fungi and the mechanisms of fungal virulence. This information would identify many

new potential drug targets and could be applied to the design of more sophisticated assays for antifungal screening.

There are extensive differences between fungi and mammals that are yet to be exploited in the development of antifungal therapy. Fungi and humans are united in their eukaryotic origins. They have in common many cell functions essential to growth and proliferation. However, evolutionary divergence within these fundamental processes has created differences sufficient to impart fungal-specific drug interactions. Several antifungals currently in clinical use capitalize upon such differences. Imidazole and triazole compounds such as ketoconazole and fluconazole interact with and inhibit the enzyme lanosterol 14α-demethylase, thus blocking fungal sterol biosynthesis. Allylamines, e.g., naftifine and terbinafine, similarly interfere with sterol biosynthesis by inhibiting squalene monooxygenase. Despite the presence of analogous enzymes in mammalian cells, these compounds are fungal-specific inhibitors. Many essential cellular functions common to fungi and mammals are already known and there are likely many as yet undiscovered. Each has the potential to serve as a fungal specific target. Unfortunately, most studies of these processes have been conducted in non-pathogenic fungi and the critical differences intrinsic to the pathogenic species are unknown. This is due, in part, to the intrinsic difficulties in studying the molecular aspects of pathogenic species. Efforts to develop our knowledge of fundamental processes in pathogenic fungi may provide a wealth of useful information and could proceed rapidly, abetted by the tools and information obtained from nonpathogens.

Despite the similarities amongst eukaryotes, a few unique attributes of fungal cells have been identified and these could provide ideal targets for antifungal agents. The desirability of targeting fungal-specific processes stems from the presumably greater likelihood of achieving fungal specificity in drug interactions and reduced likelihood of potential toxicity to the human host. This therapy has been partially realized with one of the most effective antifungals, amphotericin B. Amphotericin B therapy takes advantage of the biochemically distinct sterol composition of the fungal membrane which contains ergosterol, the counterpart of mammalian cholesterol. Although it binds preferentially to ergosterol, partial binding to cholesterol imparts significant toxicity to amphotericin. Another unique aspect of fungi is the reliance of the fungal protein synthetic machinery on an auxiliary protein, translation elongation factor three, which has yet to be developed as a drug target.

The most overt distinction between fungal and mammalian cells is the cell wall of fungi. The uniqueness of this structure, coupled with the known utility of cell wall inhibitors in the treatment of bacterial diseases, has focused considerable attention on the wall as a premier target of antifungal drugs. The cell wall contains three major polysaccharides: mannan, ß-1,3 and ß-1,6 linked glucans, and chitin. Two classes of compounds have been found that inhibit chitin synthesis, the polyoxins and nikkomycins. Although nikkomycins exhibit anti-fungal activity *in vitro*, they have not yet been tested in clinical trials. Polyoxins have proved ineffective *in vivo*, but have found utility as an antifungal in the agricultural sector. More promising data have been obtained with inhibitors of ß-1,3 glucan synthesis. These include the papulocandins and echinocandins and there appears to be clinical potential.

While the gross composition and structure of the cell wall are fairly well defined, much remains unknown. The mechanisms that integrate synthesis of the various wall polymers, cross-link them into a cohesive structure, and remodel the wall as it expands, are as yet undefined. Also unknown are the regulatory systems needed to coordinate wall synthesis with the cell cycle and cell proliferation. The molecular components of each of these processes are likely to be unique and fungal-specific and their investigation represents an opportunity to define new potential antifungal targets. Our naiveté regarding even the gross structure and function of the fungal cell wall is evident in the results of genetic analysis of chitin function in the cell wall of *C. albicans*. Mutants lacking the gene for chitin synthase 3, which synthesizes the bulk of the chitin in the lateral cell walls, exhibited normal growth rates and no alterations in cell shape or the ability to transit between yeast and hyphal cell morphologies. Nonetheless, these mutants were greatly

attenuated in their virulence. Loss of virulence was not due to a failure of the mutant to colonize or proliferate *in vivo*. Thus, it is clear that the process of cell wall biosynthesis likely contains a plethora of exploitable targets as yet unrealized.

Since relatively few fungal species are human pathogens, these species must have distinctive attributes that impart the trait of virulence. The molecular components comprising these attributes provide additional points of therapeutic intervention. The development of drugs that specifically interfere with fungal virulence determinants has been problematic due to the difficulties in defining these determinants. While a limited number of attributes have been implicated in the pathogenesis of certain species, definitive evidence has been lacking for all but a few. One problem has been the lack of adequate experimental tools. Improvements in animal models of fungal disease and the development of molecular genetic methods applicable to pathogenic fungi promises to remedy this. Another problem is the opportunistic nature of fungal pathogens. Unlike frank pathogens, opportunists do not express predominant virulence determinants that might be readily defined. Thus, the subtlety of the process itself complicates its analysis. While inhibiting virulence determinants could have value in the prophylaxis of fungal infections, it is not clear that blocking a pathogenic attribute without specific inhibition of the organism's proliferation would be of value in the treatment of acute phase infections in an immunocompromised patient.

The value of targeting virulence determinants for drug development must await a more clear definition of fungal virulence. In fact, a large part of fungal virulence may simply be the culmination of many subtle adaptations that permit proliferation and survival within specific niches of the mammalian host. A general biological principal is that organisms tend to develop adaptations that specifically suit them for survival within the unique environment of each ecological niche. Examination of the host-pathogen interactions from this ecological perspective could be of much value. As an example, a pH-regulated gene has been identified in *C. albicans* that is essential to normal cell morphology and this requirement occurs at a pH similar to the physiological pH of humans. Mutations in the gene also compromise growth. Thus, this gene may represent a unique adaptation of this fungal pathogen for proliferation in the blood and tissues of the mammalian host. This is particularly interesting since bacterial pathogens use pH, as well as other environmental stimuli, as key signals regulating the expression of virulence determinants. A significant benefit of this environmental concept is that once an important environmental signal is identified, analysis of the organism's response to that signal can identify other genetic attributes of potential importance to virulence. This may provide a viable approach to defining the subtleties of fungal virulence and the value of antifungal therapy directed toward virulence.

Finally, it should be noted that fungi have unique life cycles that include sexual and asexual spore production. A number of fungal diseases are spread via spores that germinate upon introduction into a suitable host organism. Inhibitors of fungal spore germination would, therefore, be a useful prophylactic therapy for at risk patients such as those with AIDS. Although spore germination is a complex and highly regulated process, substantial progress has been made in identifying key regulatory molecules. These molecules are potential fungal-specific targets for germination inhibitors.

Recommendations

- Encourage the application of the present knowledge base of the molecular mechanisms of cell growth and proliferation to pathogenic fungi.
- Encourage more detailed studies of fungal-specific attributes such as cell wall biosynthesis.
- Encourage studies to delineate the basis of fungal virulence.
- Promote academic-industrial partnerships to investigate and exploit potential antifungal targets.

IMMUNOLOGIC APPROACHES AND METABOLITE DETECTION

Current Status and Limitations

Despite the fact that the frequency of infections caused by fungi has increased markedly in recent decades, our ability to recognize these infections early is quite limited. Often, diagnosis by isolation of the causative fungus and its subsequent identification is unsatisfactory because the pathogen of interest (i) may not grow axenically (e.g., *Pneumocystis carinii*), (ii) is cultivable in only a minority of infected individuals (e.g., *Candida* or *Aspergillus*), or (iii) grows too slowly to provide timely diagnostic information (e.g., *Histoplasma*). Therefore, alternative methods for demonstrating that an individual has a serious fungal infection have been investigated.

Immunological methods have long been used to demonstrate humoral immune responses to fungal antigens, and some of these responses have clear diagnostic significance. For example, presence of complement-fixing antibodies to *Coccidioides immitis* in the blood or cerebrospinal fluid usually implies presence of active infection. In other mycoses, however, serologic testing for antibodies is less useful. For example, antibodies to fungi such as *Candida* and *Aspergillus* are detectable in the serum of healthy individuals, so these antibodies clearly do not imply presence of serious infection. Also, the immunocompromised patients who are most likely to develop opportunistic mycoses are precisely those who are least able to mount diagnostic humoral immune responses. Lastly, antigenic cross reactivities between different fungi limits the diagnostic usefulness of serologic testing. One promising approach to improving the diagnostic accuracy of serologic testing for fungal infections has recently been explored with *Blastomyces dermatitidis*. A 120 kDa protein antigen, WI-1, has been identified, the gene cloned and an immunodominant 25-amino-acid repeat from this protein has been expressed. WI-1 shares immunologic determinants with the A antigen of *B. dermatitidis* (a commercially-available glycoprotein antigen). However, whereas A antigen cross-reacts with *Histoplasma capsulatum* antigens, WI-1 antigen does not. Furthermore, monoclonal antibodies to the recombinant 25-aa repeat abolished binding of anti-A antisera to A antigen. These results suggest that immunologic cross reactivities between glycoprotein antigens of different fungi may be due to similarities in their polysaccharide moieties and that it may be possible to circumvent this problem by producing nonglycosylated recombinant protein antigens in prokaryotic expression systems.

Immunologic methods also can be used to demonstrate fungal antigens, and this approach has been used in several diagnostic tests. For example, immunoassays for the capsular polysaccharide of *Cryptococcus neoformans* are very accurate, and immunoassays for *Candida* and *Aspergillus* antigens also have been described. Unfortunately, there is little standardization between various methods, and results obtained in different laboratories are seldom comparable. One promising approach to generating well-characterized and standardized reagents is being pursued to examine the structural determinants that influence binding of monoclonal antibodies (mAbs) to *C. neoformans* glucuronoxylomannan (GXM). Molecular modeling suggested that a tryptophan-101 of the light chain of mAb 439 is within the GXM binding pocket, and this hypothesis was supported by (i) conservation of a tryptophan in this position among multiple anti-GXM light chains and (ii) by a predicted shift in fluorescence emission when GXM was added to mAb 439. Also, presence of five basic amino acids in the vicinity of the putative binding site in mAb 439 suggests that strong charge interactions influence antibody binding to GXM, and this hypothesis is supported by a reduction in binding of antibody to GXM by carboxyl reduction of GXM. These studies show that systematic studies of the structural determinants of antibody binding to fungal antigens such as *C. neoformans* GXM may lead to the rational design of antibodies ideally suited for detecting and quantifying antigens of *C. neoformans* and other fungi.

A third way to diagnose fungal infection is to demonstrate a distinctive fungal metabolic product in the body fluids and/or tissues of an infected host. It has been shown that several pathogenic fungal species produce large amounts of acyclic polyols and that these polyols can be used as diagnostic markers. For example, several pathogenic *Candida* species produce large amounts of

the 5-carbon polyol D-arabinitol, and animals and humans with invasive candidiasis have elevated serum D-arabinitol/creatinine ratios. Furthermore, several species of *Aspergillus* and *C. neoformans* produce large amounts of the 6-carbon polyol mannitol in culture, and animals with invasive aspergillosis and cryptococcosis have high body fluid and/or tissue mannitol levels. However, this diagnostic approach is seldom used in clinical practice, principally because it requires the availability of complex analytical methods such as multidimensional gas chromatography (GC) and combined GC/mass spectrometry. A more practical alternative method for measuring D-arabinitol in body fluids has recently been developed. The D-arabinitol dehydrogenase (ArDH) of *Candida tropicalis* catalyzes the reaction D-arabinitol + NAD => D-ribulose + NADH with a high degree of substrate specificity. An automated enzymatic assay for D-arabinitrol in human serum was developed using ArDH as the key reagent, and a large prospective clinical study established the usefulness of serial D-arabinitol measurements in neutropenic cancer patients. Lastly, the gene encoding arDh has been cloned and overexpressed in *E. coli*, and a simple method for purifying large amounts of the recombinant enzyme has been described. Thus, it is technically feasible to use serial automated D-arabinitol testing in clinical practice. Nevertheless, this technology is not yet available to clinicians because it does not yet have a corporate developer.

Recommendations

- Encourage the development of new and/or improved methods for diagnosing fungal infections by demonstrating specific humoral immune responses.
- Encourage the development of new and/or improved methods for diagnosing fungal infections by demonstrating specific fungal antigens.
- Encourage exploration and/or development of nontraditional diagnostic approaches, such as detection and quantization of distinctive fungal metabolites.
- Actively promote and facilitate interactions between government, academia and industry to encourage commercialization of promising diagnostic tests. This is especially important in cases in which large companies may perceive markets to be small.
- Encourage the incorporation of diagnostic test evaluation into ongoing therapeutic and epidemiologic studies.

MOLECULAR APPROACHES AND NEW DIAGNOSTIC TEST DEVELOPMENT

Current Status

Despite recognition of the clinical importance of fungal infections, these infections remain difficult to diagnose and treat. In recent years, the clinical diagnosis has been enhanced by the recognition of newer clinical presentations and the definition of several major independent risk factors. Likewise, the laboratory diagnosis has been improved by newer blood culture methods, and by developments in the areas of serologic and molecular diagnostic testing. Given the limitations of conventional diagnostic methods, it is not surprising that microbiologists, mycologists, and infectious disease clinicians have looked to the newer techniques of modern molecular biology as additional, and perhaps more definitive, means of diagnosing fungal infections and characterizing fungal species. By combining the methods of classical microbiology and molecular biology, new light has been shed upon fungal genetics and the interrelationship of fungi as well as antifungal susceptibility and molecular mechanisms of resistance to antifungal agents. As these efforts provide new insights into the diagnosis and therapy of fungal infections, existing collaborative groups such as the NIAID Mycoses Study Group and others will serve as a vehicle to help translate the more fundamental discoveries into clinical reality.

In the last decade, the techniques of molecular biology have been applied to the study of fungi and have allowed advances both basic and clinical. With the development of PCR, researchers began asking what fungi are and what they are most closely related to. Phylogenetic questions

regarding both pathogenic and nonpathogenic fungi are being addressed by determining gene sequences for the 18S rRNA subunit in many species. This approach allows one to identify sequences that are conserved among all fungi and by which fungi are distinguished as a group from other eukaryotes. A major benefit from this research is the identification of sequences that may be useful in a PCR assay and as specific probes for detection and identification of fungi. Primers and probes, based on 18S rRNA genes, are now available to amplify fungal DNA at the level of all fungi (universal), genus, species, or even sub-species. This approach allows investigators to examine large numbers of different organisms and to pursue basic questions about species and pathogenicity as well as provide tools for clinical application. The identification of polymorphic loci allows investigators to build taxonomic trees that can be used in a logical manner to characterize new and established fungal pathogens. One of the major challenges in the field of mycology is to maintain and update the ever expanding molecular data base related to fungi.

Certainly one of the most attractive aspects of these molecular approaches is the potential for clinical application. The use of PCR and molecular probes for detection and identification of fungal pathogens in clinical material is clearly very exciting. One caution that must be kept in mind is that the primer or probe does not define the fungus. One must still apply the techniques of classical mycology and consider all available information in arriving at the correct identification of an organism. Another major concern regarding the application of molecular methods in diagnosis of fungal infection is the lack of adequate sensitivity. This has been an issue in the few clinical studies designed to use molecular methods to detect fungi in clinical material and may stem from the low numbers of organisms present in the specimen and the problems in extracting adequate DNA from fungi. Regardless of the problems and pitfalls encountered, molecular approaches promise to expand our understanding of fungi and to improve our diagnostic capabilities.

The available laboratory methodology in directing antifungal therapy has been evolving. In the past ten years, considerable effort has gone into developing standardized methods for antifungal susceptibility testing. Groups such as the NCCLS Subcommittee for Antifungal Testing have carefully identified the variables responsible for poor reproducibility of *in vitro* susceptibility testing and now have recommended a standardized reference broth macrodilution method. Current efforts are directed at developing simpler more user-friendly methods such as broth microdilution and agar diffusion and towards establishing the clinical validity of such testing.

With the increasing frequency of fungal infections world-wide, considerable attention has been directed towards various aspects of antifungal therapy. In general, resistance to antifungals thus far has been less of a problem than has bacterial resistance to antibacterials. However, recent experiences of antifungal drug treatment failures combined with improvements in performance and standardization of antifungal susceptibility testing have drawn attention to the problem of antifungal resistance. Given the broad use of fluconazole and other azoles, resistance to these agents among clinical isolates of *Candida* species has received the most attention. Three types of drug-resistance effects seem to occur: (1) replacement of an initially susceptible species (*C. albicans*) by an intrinsically resistant species of *Candida* (*C. krusei* or *C. glabrata*); (2) replacement of an initially susceptible Candida strain by a more resistant strain of the same species; and (3) development of resistance in a single strain (genotype) of *Candida* species.

Our understanding of the mechanisms of antifungal resistance lags far behind our knowledge of antibacterial resistance mechanisms. With the recognition of azole resistance, it has become apparent that several approaches used successfully in studying other eukaryotic and prokaryotic systems may be applicable to studying drug resistance in fungi. Efforts have focused on studying point mutations in drug targets, active efflux mechanisms such as multi-drug resistance genes (MDR) that pump drugs out of cells, and target gene amplification. In each case, examples of specific resistance mechanisms have been found in clinical isolates of fungi.

The mechanism of action of azole antifungals is reasonably well understood and involves inhibition of cytochrome P450-dependent enzymes such as 14 demethylase (14DM) resulting in disturbed membrane stability and altered function of membrane-associated enzymes. Recognized mechanisms for azole resistance include reduced intracellular accumulation of drugs, due to either decreased uptake or increased efflux (MDR), altered 14 DM or other ergosterol biosynthetic enzymes, and amplification of genes (CYP51) encoding for target enzymes with resultant overproduction of the drug target. In fact, all of these mechanisms have been identified in one or another series of isolates of *Candida* obtained from patients failing azole therapy. Recognition of parallel resistance mechanisms in other cell systems may facilitate the study of antifungal resistance. Additional work is necessary to further our understanding of antifungal drug resistance. This knowledge will aid in our efforts to design new drugs and to control the spread of resistance.

Recommendations

- Expand the development of molecular diagnostics for fungal infections.
- Encourage the use of nucleic acid probe and PCR-based methods for fungal detection and identification.
- Encourage more interaction at the basic/clinical research interface regarding fungal infections.
- Encourage studies of basic mechanisms of action and resistance to antifungal agents using experimental systems that incorporate both fungi and other eukaryotic microorganisms.
- Develop physical maps of the fungal genome(s).
- Encourage academic-industrial-government partnerships for develop-ment of new fungal diagnostic tests.
- Make further development and evaluation of promising new fungal diagnostic tests a priority at the federal (NIH) level.

BIOLOGIC RESPONSE MODIFIERS WITH THERAPEUTIC POTENTIAL

Current Status

Experimental infection of inbred mice with *Leishmania major* has been a valuable model for determining the role of $CD4^+$ subset development during the progress of infection. Studies with this model system will likely provide insight into the immunological response during infections with the fungal organisms, since it is known that $CD4^+$ lymphocyte progress development plays an important role in defense against certain *fungi* organisms.

The macrophage is a key cell for host defense against *Leishmania*. Therefore, macrophage activating cytokines are essential components of host defense. For the production of these macrophage activating cytokines, a robust Th1 cell response is necessary. These important Th1-derived cytokines include IFN-g, IL-2, IL-4, and IL-7. The $CD4^+$ phenotype switch that results in the dominance of either the Th1 or Th2 subsets is dependent upon the presence of certain cytokines during the time of initial priming of T cells. These cytokines are IFN-g, IL-12, and IL-4. IL-12 mediates Th1 proliferation and IL-4 mediates Th-2 proliferation (IL-10 for Th2 cells and IFN-gfor Th1 cells may be important in certain inbred mice). BALB/c mice are highly susceptible to *Leishmania major* infection, since their primary response to infection is a Th2 dominant response that is both unable to activate macrophages and actively blocks the action of Th1-derived cytokines. Accompanying their Th2 response is a high level of IL-4 production. Of interest is that IL-12 given to mice susceptible to *Leishmania major* with soluble *Leishmania* antigens has resulted in immunoprotection of these mice to subsequent infection with *Leishmania major*.

A clearer understanding of the importance of the Th1 response in defense against *Leishmania major* infection has evolved from the analysis of the susceptibility of this inbred strain to infection by this intracellular parasite. Identifying which subset of $CD4^+$ derived lymphocytes is important in defense of a specific infection is critical to identifying antigens for the T cell subset that might become potent and specific antigens for vaccine development. Additionally, development of strategies for using specific cytokines with antigens to specifically activate T cell clones is likely possible. Studies paralleling the work on this model system of Leishmania infection are already underway for fungal diseases.

Lymphocytes comprise a critical component of host defenses against cryptococcosis. Freshly isolated T and NK cells are conjugate with, and directly inhibit the growth of *Cryptococcus neoformans*. During *in vitro* culture, the fungistatic capacity of human lymphocytes is lost unless the cells are activated with the cytokine interleukin-2. Antifungal activity appears to be a receptor-mediated event, and lymphocyte-mediated fungistasis is markedly diminished if surface receptors on peripheral blood mononuclear cells (PBMC) are cleaved by treatment with the proteases trypsin or bromelin.

Tumor necrosis factor-α (TNF-α) is a key mediator of inflammation and may promote HIV replication in latently infected cells. Cryptococcosis often is associated with aberrations in the host inflammatory response and occurs preferentially in persons with AIDS. Recent studies have defined the conditions under which human leukocytes produce TNF-α, when stimulated by *C. neoformans.* Peripheral blood mononuclear cells (PBMC) produced comparable amounts of TNF-α following stimulation with *C. neoformans* and lipopolysaccharide (LPS). Detectable TNF-α release in response to *C. neoformans* occurred only when fungi with small-sized capsules were used and complement-sufficient serum was added. Fractionation of PBMC established that monocytes were the predominant source of TNF-α. TNF-α gene expression and release occurred significantly later in PBMC stimulated with *C. neoformans* compared with LPS. *C. neoformans* was also a potent inducer of TNF-α from freshly isolated bronchoalveolar macrophages (BAM). Upon *in vitro* culture, BAM and monocytes bound greater numbers of fungal cells yet their capacity to produce TNF-α following cryptococcal stimulation declined by 74% - 100%. However, this decline was reversed if the BAM and monocytes were cultured with interferon g. These data establish that *C. neoformans* can potently stimulate TNF-α release from human leukocytes. However, several variables profoundly affect the amount of TNF-α released, including the type of leukocyte and its state of activation, the size of the cryptococcal capsule, and the availability of opsonins.

Deactivation of mononuclear phagocytes is critical to limit the inflammatory response but can be detrimental in the face of progressive infection. Studies have compared the effect of the deactivating cytokine IL-10 on human PBMC responses to LPS, *Cryptococcus neoformans* and *Candida albicans.* IL-10 affected dose-dependent inhibition of TNF-α release in PBMC stimulated by LPS and *C. neoformans*. In contrast, even at doses as high as 100 U/ml, IL-10 inhibited TNF-α release in response to *C. albicans* only by 50%. IL-10 profoundly inhibited release of IL-1ß from PBMC stimulated by all three stimuli. TNF-α mRNA and release ßmRNA was of lesser magnitude and occurred only when IL-10 was added within 2 h of cryptococcal stimulation. IL-10 inhibited translocation of nuclear factor kB in response to LPS, but not the fungal stimuli. All three stimuli induced IL-10 production in PBMC, although over ten-fold less IL-10 was released in response to *C. neoformans*.

Thus, while IL-10 has deactivating effects on PBMC responses to all three stimuli, disparate, stimulus and response specific patterns of deactivation are seen. Inhibition by IL-10 of proinflammatory cytokine release appears to occur at the level of gene transcription for TNF-α and both transcriptionally and post-transcriptionally for IL-1ß. These important studies will likely add to strategies for enhancing the immune response against *Cryptococcus* and other fungi.

The role of natural antibody immunity in host defense against fungi is uncertain despite several decades of intensive study. It has been difficult to consistently correlate protection against infection with the presence of serum antibody. For *Cryptococcus neoformans* there are many published observations providing evidence for and against a role for antibody immunity. However, in recent years three independent groups have been demonstrated to mediate protection in murine models. These observations are important because they suggest that production or administration of protective antibodies in humans could be useful in prevention or treatment of *C. neoformans* infection.

The availability of mAbs has permitted the evaluation of the protective efficacy of *individual* antibodies. Remarkably, it was discovered that the IgG3 isotype mediated no protection in mice whereas the IgG1 resulted in conversion of a non-protective mAb into a protective mAb establishing a critically important role for constant region function in protection. The identification of protective and non-protective antibodies provide a potential explanation for the difficulties associated with demonstrating a role for natural antibody immunity in protection. Presumably, the protective efficacy of natural antibody responses reflects the relative proportion of protective and non-protective antibodies in serum.

Comparison of 2 IgM antibodies to GXM derived provided a compelling demonstration that fine specificity was also crucial for protective efficacy. These two IgMs were derived from a single B cell but differed by several amino acids in their antigen binding domains which are the result of somatic mutations during the generation of the immune response. One IgM produced a circular indirect immunofluorescence pattern after binding *C. neoformans* and was protective. The other IgM produced a "speckled" pattern by indirect immunofluorescence and was not protective.

In vitro studies suggest several mechanisms by which antibodies modify the course of *C. neoformans* infection in mice. MAbs to the capsular polysaccharide are potent opsonins and enhance the antifungal efficacy of macrophages. Immune complexes composed of polysaccharide and antibody can enhance nitric oxide production in murine macrophage cells. *In vivo* studies have shown that antibody administration results in a rapid decline of serum antigen levels. Since antigen has been associated with a variety of deleterious effects, antibody-mediated clearance of capsular antigen could contribute to protective effects.

Molecular studies of anti-*C. neoformans* antibodies have shown that antibody response to *C. neoformans* is highly restricted in terms of variable gene utilization. Antibodies using the same variable gene combinations can be either protective or non-protective depending on minor differences in primary structure resulting from somatic mutations. The identification of protective antibodies for *C. neoformans* is an exciting development because it suggests that antibodies can be exploited for prevention and therapy of infection *even* if the role of natural antibody immunity is uncertain. Vaccines may be developed which elicit protective antibody immunity and the presence of antibody may enhance cellular defenses to prevent the establishment of infection. Alternatively, protective antibodies can be used directly as therapeutic drugs as was done in the preantibiotic era. MAbs to *C. neoformans* have been shown to enhance the efficacy of amphotericin B and fluconazole (15) *in vitro* and *in vivo*. This paradigm, if proven in practice could be applicable to other pathogenic fungi.

Recommendations

- Utilize lessons learned from study of other eukaryotic pathogens such as *Leishmania* to study pathogenesis of intracellular fungal pathogens such as *Histoplasma.*
- Expand efforts to evaluate efficacy of vaccines for certain fungal infections.
- Continue and expand investigations of the role of passive immunotherapy for fungal infections, especially cryptococcosis, histoplasmosis, and coccidioidomycosis.

DRUG DISCOVERY

Current Status

The purpose of the session was to provide an overview of the drug-discovery process in private industry with an emphasis on the description of specific mechanisms of interest and how methods are adapted for the screening process.

In the early 1970's, when the number of world-wide patents numbered less than 100 per year, the introduction and subsequent success of the azoles spawned a huge increase in activity in the antifungal drug discovery process across the industry such that by the mid-70's the annual number of world-wide patents exceeded 400, and by 1993 numbered greater than 700. In 1973, the patent literature for antifungals was dominated by Japanese companies, but by 1983, four of the top five companies (in terms of numbers of patents filed) were European or American. By 1993, only a single Japanese company was in the top ten of companies filing patents.

With respect to trends in the types of antifungal compounds patented, the 1970's saw dramatic increases in the number of synthetics, with azoles accounting for much of this activity. Although azoles continue to be an important component of the patent literature, natural products continue to be an important source for drug discovery. An important sector in more recent years is represented by semi-synthetics, with compounds that originated as natural products being improved through a medicinal chemistry process that has sought to make improvements in potency, toxicity profile, or solubility characteristics.

An overview of the antifungal discovery program based at pharmaceutical companies was presented through three representative examples: Shaman Pharmaceuticals, Abbott Laboratories, and Merck. Shaman's program is based on an ethnobotanical approach, screening plants that have been used by indigenous ethnic groups for treatment of various infectious diseases. The program pays particular attention to the plant parts indicated by the local shaman, and often emulates, in an approximate fashion, the methods used by these traditional healers in the preparation of extracts and elixirs. Information on activities seen at the extract level was presented, and demonstrated a high percentage of activities, particularly against dermatophytic fungi.

At present, one of the directed screens of Abbott involves the search for inhibitors of fungal topisomerase I and II. Because of identifiable differences in the topisomerase enzymes of *Candida* and mammals, Abbott's screening program is conducted with the hopes of finding inhibitors that will have a differential activity vs. the fungal enzyme, while having a favorable toxicology profile. To date, some 50,000 compounds from various libraries have been screened, with small numbers of compounds exhibiting the differential activity identified.

Merck continues to have a broad approach to their screening program, with a heavy reliance on the screening of natural products from a variety of sources. Such screening has led to the discovery of the pneumocandins: potent inhibitors of fungal glucan synthase. This class of compound has shown fungicidal activity vs. *Candida*, and may also be useful in fighting infections due to *Aspergillus* and *Pneumocystis*. The identification and exploitation of screens that focus on unique targets may offer new opportunities for drug discovery. Using this diversity of approaches in recent years, Merck has had a dominant position in the number of antifungal patents filed.

The underlying theme expressed by the participants is that industry is striving to discover new classes of compounds, both through the screening of new materials and by the use of new mechanistic assays.

Recommendations

- The continued funding of basic research that will help identify new targets will ultimately be of benefit in the screening for new antifungals.
- Participation by the academic sector in both basic and applied research, and formation of partnerships with private industry should be encouraged.

PRIVATE, PUBLIC AND ACADEMIC PARTNERSHIPS AND ANTIFUNGAL DRUG DEVELOPMENT

Current Status

The development process for antifungal agents has enjoyed only a modest commitment of resources, and hence has been limited to the extent to which the academic community, industry, and governmental agencies have participated in cooperative efforts. As the need increases for the development of succeeding generations of antifungal agents, resources appear all the more constrained. Therefore, it is critical that thought be given to the challenge that these realities present and that a clear agenda be set to direct future efforts.

The epidemiology of mycotic infections has changed dramatically over the last one to two decades, owing to the advent and continuing expansion of the HIV epidemic, progress in the clinical management of immunocompromised patients, and the emergence of strains of fungi that are becoming increasingly resistant to greater numbers of agents in the clinician's armamentarium.

In partial response to these pressures, clinical researchers have tapped into mainstream management practices of bacterial infections, such as combination therapy and prophylaxis. Clinical research has been a strength in this area, with well-developed collaborative research groups. There remain potential gains through improving efficiencies, such as in standardized clinical trial designs, and through innovations, such as randomized Emergency INDs and use of large, simple trials.

The emergence of resistant strains and the greater use of combination therapy have, in turn, historically brought greater attention to the development of *in vitro* methods to measure susceptibility. The U.S. Food and Drug Administration, as well as other regulatory bodies, recognizes the importance of giving timely regulatory direction to the development of test kits for commercial use. This prospective approach to providing direction is reflective of a heightened awareness of the importance of collaboration among all of the members of the medical community. Strategies to focus additional efforts should extend and consolidate many of the present efforts to develop infrastructure and professional careers.

The National Institute of Allergy and Infectious Diseases has contributed substantially towards creating an environment that provides opportunities to develop partnerships in basic and clinical research with individual investigators, industry, and government. Examples include traditional and innovative mechanisms, such as informal professional collaborations, co-funding mechanisms, CRADAs, facilitating the use of Small Business Innovation Research (SBIR) grant applications, and the sponsorship of professional meetings for the exchange of information.

Mycology is enjoying a greater awareness by the scientific and medical communities, in general. It is in everyone's interest - the private sector, government, and the academic communities - to capitalize on this interest and bring greater degrees of cross-fertilization to all of the disciplines. New directions should enhance the availability of evolving knowledge across disciplines.

Recommendations

- Construction of an electronic bulletin board that networks the human, veterinary, and plant mycology literature. Inter-disciplinary support should also extend to the posting and sponsorship of presentations and meetings.
- Linking resources, either through modern means of communications or through centralization, to facilitate the spectrum of mycology research related to antifungal drug development to include:

- epidemiology, for tracking evolving resistance patterns;
- sera and tissue banks, for unknowns in *in vitro* testing;
- genomic sequences, for taxonomic research; and
- informatics, for designing new therapeutic agents and for following trends and cross-referencing bibliographies.

- Fostering career opportunity awareness at the undergraduate level, for example, through course offerings, guest lectureships, and summer programs for research and demonstration projects.
- Fostering opportunities for mid-career professionals to learn and cross-train quickly and efficiently in new fields or with new technologies.

TOPIC SUMMARY AND SPEAKERS

Theme Lecture: Status of Diagnosis and Treatment

William E. Dismukes, University of Alabama

Targets for Inhibiting Proliferation or Virulence

Chairperson: William Timberlake, Myco Pharmaceuticals
Christophe D'Enfert, Institut Pasteur
William Fonzi, UC, Irvine

Immunologic Approaches and Metabolite Detection

Chairperson: Brian Wong, University of Cincinnati
Bruce Klein, University of Wisconsin
Tom Kozel, University of Nevada

Molecular Approaches and New Diagnostic Test Development

Chairperson: Michael Pfaller, University of Iowa
John Taylor, UC, Berkeley
Mahmoud Ghannoum, Harbor-UCLA
John Galgiani, VAMC, Tucson
Ted White, UCSF

Biologic Response Modifiers with Therapeutic Potential

Chairperson: John E. Edwards, Jr., Harbor-UCLA
Richard Locksley, UCSF
Stuart Levitz, Boston University
Arturo Casadevall, Albert Einstein

Drug Discovery

Chairperson: Richard Hector, Shaman Pharmaceuticals
Paul Lartey, Abbott Laboratories
Myra Kurtz, Merck

Private, Public and Academic Partnerships

Chairperson: Paul Beninger, FDA
Doug Webb, Pfizer
Michael McNeil, CDC
Steve Gutman, FDA
Steve Gitterman, FDA
Dennis M. Dixon, NIH
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