CLINICAL UTILITY AND DEVELOPMENT OF BIOMARKERS IN INVASIVE ASPERGILLOSIS

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ABSTRACT

The diagnosis of invasive aspergillosis remains very difficult, and there are limited treatment options for the disease. Pre-clinical models have been used to evaluate the diagnosis and treatment of *Aspergillus* infection and to assess the pathogenicity and virulence of the organism. Extensive efforts in *Aspergillus* research have significantly expanded the genomic information about this microorganism. The standardization of animal models of invasive aspergillosis can be used to enhance the evaluation of genomic information about the organism to improve the diagnosis and treatment of invasive aspergillosis. One approach to this process has been the award of a contract by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health to establish and standardize animal models of invasive aspergillosis for the development of new diagnostic technologies for both pulmonary and disseminated *Aspergillus* infection. This work utilizes molecular approaches for the genetic manipulation of *Aspergillus* strains that can be tested in animal-model systems to establish new diagnostic targets and tools. Studies have evaluated the performance characteristics of assays for cell-wall antigens of *Aspergillus* including galactomannan and beta-D-glucan, as well as for DNA targets in the organism, through PCR. New targets, such as proteomic and genomic approaches, and novel detection methods, such as point-of-care lateral-flow devices, have also been evaluated. The goal of this paper is to provide a framework for evaluating genomic targets in animal models to improve the diagnosis and treatment of invasive aspergillosis toward ultimately improving the outcomes for patients with this frequently fatal infection.

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Invasive fungal infections remain a major source of morbidity and mortality, especially in immunosuppressed patients (1). Outcomes of these infections are significantly improved if the diagnosis is established early and antifungal therapy is begun promptly. Unfortunately, cultures of blood and other body fluids for many of these pathogens, are not often positive, and invasive procedures to establish a tissue diagnosis are complicated by the frequent presence of thrombocytopenia and neutropenia in patients at risk for infection with these pathogens. Thus, extensive efforts have been undertaken to establish non-culture-based methods for the diagnosis of invasive mycoses.

Biomarkers have been developed to diagnose a variety of invasive fungal infections (Table 1). Serologic testing for antibody can be useful to establish prior exposure to the causative pathogens, but is usually not specific enough to establish a diagnosis of invasive infection. In the case of some endemic infections, such as coccidioidomycosis, antibody testing is useful for the diagnosis of active disease as well as for following the response to therapy (2). For opportunistic infections like aspergillosis, antibody is often present from prior exposure, but for immunosuppressed patients seroconversion rarely occurs even with extensive disease (3).

Thus, biomarker assays have focused on antigens or nucleic-acid detection methods that are positive in patients with invasive fungal infections. In some of these infections, such as cryptococcosis and histoplasmosis, the detection of antigen as a marker of invasive infection has become a standard for diagnosis and can also be important in the management of infection (4, 5). The use of biomarkers

TABLE 1
Biomarkers in Invasive Fungal Infections

Infection	Biomarker		Response to Therapy
	Antibody	Antigen	
Candidiasis	No	Investigational	No
Cryptococcosis	No	Yes	Limited
Blastomycosis	Limited	Yes	Yes (Antigen)
Coccidioidomycosis	Yes	Investigational	Yes (Antibody)
Histoplasmosis	Limited	Yes	Yes (Antigen)
Paracoccidioidomycosis	Yes	Yes	Unknown
Aspergillosis	No (for invasive disease)	Galactomannan; 1,3-beta-D-glucan; Nucleic acid	Limited
Zygomycosis	No	Investigational	No
Other moulds	No	Investigational	No

in these infections has yielded important knowledge in developing biomarkers for the diagnosis of invasive fungal infection. As an example of this the importance of understanding the characteristics of a biomarker is demonstrated with cryptococcal capsular polysaccharide antigen. The capsular polysaccharide antigen of *Cryptococcus* is usually present in both serum and cerebrospinal fluid (CSF) in patients with active infection, but because it is not rapidly cleared, high titers of the antigen may persist, especially in serum and even with effective therapy. On the other hand, dramatic changes in cryptococcal antigen titers can be useful in following the response to therapy. For example, serial cryptococcal antigen titers might be useful in distinguishing progressive infection, with rising titers, from immune reconstitution inflammatory syndrome (IRIS), in which titers would be low (6).

Detection of the polysaccharide antigen of *Histoplasm* demonstrates the impact of host immune status as well as the utility of serial antigen measurement in assessing disease (5). Normal hosts and non-HIV infected patients have limited positivity for this biomarker, perhaps reflecting the extent of infection. In contrast, the test for this biomarker in patients with advanced AIDS and widely disseminated histoplasmosis can very sensitively and specifically establish the diagnosis in specimens of urine, blood, and other body fluids. Serial measurement of this antigen has become very useful in the monitoring response to therapy and in predicting relapse in histoplasmosis.

BIOMARKERS IN INVASIVE ASPERGILLOSIS

Non-culture-based methods have been used to establish a rapid diagnosis of invasive aspergillosis. Most of these methods have been targeted to *Aspergillus* antigens or metabolites, and more recently to molecular targets associated with invasive infection by the organism.

Detection of galactomannan in serum and more recently in other body fluids, especially bronchoalveolar lavage (BAL) fluid, has played an important role in the non-culture-based diagnosis of invasive aspergillosis (7). The current enzyme immunoassay (EIA) (Platelia *Aspergillus*; Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France; Bio-Rad, Redmond, WA) utilizes a monoclonal antibody to galactomannan and has been extensively validated in experimental models and in clinical studies (7). The sensitivity of the assay for diagnosing invasive aspergillosis has reportedly been as high as 89%, with a specificity of 92%, in high-risk patients having hematopoietic stem-cell transplants (8). More recent studies have found a lower sensitivity, ranging from

40% to 50%, particularly in the setting of mold-active antifungal agents and with nonserial testing, so that a positivity index of 0.5 is now recommended in high-risk patients (9–11). False-positive results have been seen in some patients, including neonates, which may be due to the presence of cross-reacting antigens from bacteria such as *Bifidobacterium* in patients receiving antibiotics, particularly piperacillin-tazobactam, as well as in some other fungal infections such as histoplasmosis (7, 12–14).

The use of PCR to establish a molecular basis for diagnosis has also been evaluated in invasive aspergillosis, but a number of features still limit its utility (15, 16). There are no externally validated methods for such PCR-based diagnosis, and a variety of targets and techniques have been utilized (17). A recent meta-analysis of published results of PCR studies showed a sensitivity of 75% and specificity of 87% when 2 positive samples were used to establish a positive result; for a single sample, this rose to a sensitivity of 88% at the cost of a reduced specificity of 75% (18). Recent efforts have focused on optimizing methods for extracting DNA from clinical samples and in standardizing the process for detecting the DNA product of PCR to increase the utility of PCR-based assays in invasive aspergillosis (19, 20).

Another non-culture-based method cleared by the FDA for clinical use is based on the detection in serum of 1,3-beta-D-glucan with an limulus amoebocyte lysate test (21, 22). This assay detects cell-wall 1,3-beta-D-glucan, with the result that it is not specific for *Aspergillus* but is also positive in infections caused by other fungi having 1,3-beta-D-glucan, including *Candida* and some molds other than *Aspergillus*. Although clinical studies of the utility of the 1,3-beta-D-glucan assay in invasive aspergillosis have been limited, its is included in the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) definitions for invasive fungal infection (23).

INVESTIGATIONAL STUDIES FOR BIOMARKER DEVELOPMENT IN INVASIVE ASPERGILLOSIS

Biomarkers in other body fluids, such as BAL, can also be tested as indicators of infection, although they have not been well characterized and their utility as compared with their detection in serum has been less extensively studied (24). In one clinical study, detection of galactomannan in BAL fluid increased the diagnostic sensitivity as compared to serum for aspergillosis from 47% to 85% (24), and BAL fluid was the only sample that was positive in other studies (25, 26). False-positive results

have been reported in patients with colonization by *Aspergillus* and with the use of fluids used for BAL that contain galactomannan, such as plasmalyte (27, 28). Although the FDA has recently cleared the detection of galactomannan in BAL for clinical use, studies are being done to characterize the sensitivity and specificity of galactomannan BAL in fluid for the diagnosis of invasive aspergillosis. Pre-clinical evaluation in our established guinea pig model of invasive aspergillosis showed that BAL fluid was significantly more positive than serum, and that detection occurred more than 2 days sooner with BAL fluid than with serum, supporting the utility of BAL fluid as a clinical tool (29, 30). We also compared real-time PCR of BAL fluid with that in serum and whole blood in that same model. In that setting, as in the case of clinical infection, we showed that the PCR signal in serum or whole blood was early but inconsistent as compared to that in BAL fluid, which yielded early, consistent product detection (30).

Pre-clinical models have also been useful in characterizing effects of antifungal agents on biomarker detection. One important clinical issue has been the effect of antifungal agents on galactomannan detection, and specifically whether echinocandins (or other antifungal agents) might produce a paradoxical effect in causing an increase in galactomannan through its release from the cell wall after exposure to these agents. Galactomannan is both released by remodeling hyphae and bound to the 1,3-beta-D-glucan in the cell wall. The echinocandins inhibit 1,3-beta-D-glucan synthesis in the cell wall and could in theory potentially increase galactomannan release by reducing its binding to the cell wall or decrease galactomannan release by inhibiting fungal growth. Conflicting clinical and pre-clinical evidence supports both such effects (31, 32). We therefore used our guinea pig model of invasive aspergillosis to evaluate the effects of antifungal therapy on the detection of biomarkers of aspergillosis (29). In this model we used both single and multiple doses of an echinocandin (caspofungin), a triazole (voriconazole), and a polyene (liposomal amphotericin B). We did not find a paradoxical effect of increased galactomannan with either single or multiple doses of any of the three classes of antifungal agents tested (33). However, we did observe a consistent suppression of circulating galactomannan with both the single- and multiple-dose therapy despite very extensive infection in the lungs of all of the test animals. The suppression of circulating antigen after a single dose of each agent persisted through 4 days of observation, suggesting that in clinical settings a profound suppression of circulating galactomannan could also occur even with limited use of antifungal agents.

Serial measurement of *Aspergillus* antigen has long been recognized

to correlate with the overall outcome of infection. In studies done with Dr. Vincent T. Andriole at Yale-New Haven Hospital, we showed that clearance of *Aspergillus* antigen was associated with increased survival (8% mortality) as compared to persistent or rising antigen titers, in which case the mortality was 78% (34). To characterize the impact of antifungal therapy on survival and tissue burden, we evaluated the efficacy of posaconazole, voriconazole, amphotericin B deoxycholate, and liposomal amphotericin B in our guinea pig model. Although the extent of infection and overall survival correlated with serial measurements of antigen in these studies, it was important to recognize that extensive infection was still present despite reductions in circulating levels of either 1,3-beta-D-glucan or galactomannan (35).

Recent clinical studies have shown that the galactomannan index is a useful surrogate endpoint for outcome in invasive aspergillosis, but it is important to recognize that galactomannan may well be undetectable in early samples because of the presence of an antifungal agent, as was seen in our animal-model studies (36–38).

NEW DIAGNOSTIC TARGETS AND PLATFORMS

A number of new targets and novel platforms for biomarker detection in invasive aspergillosis are under study. For example, we evaluated a novel lateral-flow device that utilizes novel antibodies against *Aspergillus* and would potentially allow point-of-care testing (39). Other approaches have examined virulence gene products such as gliotoxin, siderophores, secreted proteases, and even volatile organic acids in breath samples (40–42). More recently, proteomic methods have been used for diagnostically identifying some pathogens, including fungi, and work has been initiated for detecting *Aspergillus* as well (43).

SUMMARY

Current non-culture-based biomarkers are effective adjuncts in the diagnosis of invasive mycoses, including invasive aspergillosis. The impact of antifungal therapy and the importance of serial testing have been evaluated in pre-clinical models and validated in clinical settings. Serial measurements of current markers for invasive aspergillosis, including galactomannan and 1,3-beta-D-glucan, correlate with outcome but should be interpreted in conjunction with other clinical indicators of infection. Novel targets and methods may further improve the diagnosis of invasive aspergillosis.

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DISCUSSION

Blaser, New York: Tom, terrific extension of your longstanding work in this field. My question is about the clinical diagnosis of aspergillosis, which, as you point out, is very difficult, I wonder if we should be aiming toward tests with increasing sensitivity, because of the issue of low numbers, local tissue infections, and trying to find the infection in systemic places like serum rather than making diagnosis easier with lateral flow or such things, which have problems with specificity. Is there any background rate of aspergillosis in uninfected people?

Patterson, San Antonio: It's an excellent question. It's not really known. Long-term colonization doesn't seem to be a typical risk factor as it is in, for instance, invasive candidiasis. Obviously, exposure occurs before infection, but detection of the organism in severely immunosuppressed patients would indicate a high likelihood of infection. One of the issues is to recognize risk, and perhaps calculate a "risk score" using a number of features, including colonization, but also, for example, genetic characteristics, as certain SNPs in toll-like receptor genes have been identified as being associated with infection. Other, more traditional risk factors, such as prolonged neutropenia, could be added to give you a certain weighted risk score. Steroid use gives you another score, and then biomarker positivity, maybe by the lateral flow method, or may be a sensitive method like PCR, gives you yet another score. You add up those scores and perhaps you get not an absolute diagnosis but a score that is associated with dramatically increased odds of the diagnosis. I think that might be an interesting way to go.

Hasday, Baltimore: I just wondered whether your BAL data were from formal BAL or mini-BALs.

Patterson, San Antonio: Well, since these are animals, they are pretty "mini," especially in the mice, but even in those small animals we actually sample the whole lung and get a good amount of sample. I think your point, though, is a good one, in that if we might be able to improve our BAL sampling, we might also improve diagnosis. Now with use of directed catheters you can sample specific regions of the lung, and that may be a way to increase sensitivity. Before, we always thought that a very peripheral lesion was very difficult to diagnose, even by BAL, but it may be that directed catheters, which you're going to know more about than me, are a way to get a sample from the location of the lesion. I think it may be a major advance, but there is not much data on it yet for aspergillosis. However, there are interesting data for increasing the yield in cancer diagnosis, and I'll make that extrapolation to aspergillosis.

Hasday, Baltimore: The other things we can get through a scope, besides fluid, are cells and cytology brush specimens, which have long been used for cancer diagnoses, and can sample the epithelium very well. My question is whether that will improve the sensitivity for organisms that stick to the epithelium.

Patterson, San Antonio: That's also a good point. I really think improved bronchoscopy technology is an important advance in this field, especially with better diagnostic markers coming on board.