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Merits and limitations of immunodiagnostic assays for systemic mycoses

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The incidence of systemic fungal diseases has increased significantly over the last decade. During that time considerable work has been done on isolating and characterizing new antigens and developing technology. However, few new immunodiagnostic tests for the mycoses have come into routine use.

Most of the currently used immunodiagnostic tests are designed to detect antibodies to specific fungal pathogens. These tests, though far from optimal, have proved useful for diagnosing aspergillosis, blastomycosis, candidiasis, histoplasmosis and other mycotic infections mainly in the immunocompetent host. They may, however, exhibit cross-reactivity, and fail to distinguish active form past infection, and colonization from invasive disease. More recently, attention has been devoted to developing antigen detection procedures. While such procedures have been successfully developed for cryptococcosis and histoplasmosis, those for the opportunistic mycoses, i.e. aspergillosis and candidiasis have been generally unsatisfactory. Their insensitivity, resulting from the transient nature of the antigen(s) detected or failure to test for a battery of diagnostic antigens. To overcome these problems, current research has focused on the use of more purified antigens, monoclonal or adsorbed polyclonal antibodies, and the refinement or introduction more sensitive assays. An overview of the immunodiagnostic tests currently used, their value and shortcomings will be presented.

Key words: Systemic mycoses, immunodiagnostic tests, aspergillosis, blastomycosis, candidiasis, histoplasmosis

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Výskyt systémových houbových infekcí se v posledním desetiletí podstatně zvýšil. V této době bylo vykonáno mnoho na izolaci nových antigenů a vývoji nových technik. Do rutinní praxe se však dostalo jen málo nových imunodiagnostických testů. Většina v současnosti užívaných testů je zaměřena na průkaz protilátek proti specifickým houbovým antigenům. Tyto testy nejsou zdaleka optimální, osvědčily se však v diagnostice aspergilózy, blastomykózy, kandidózy, histoplasmózy a dalších infekcí, zvláště u imunokompetentních hostitelů. Mohou však být ztíženy křížovými reakcemi a nejsou schopny rozlišit probíhající infekci od dříve prodělané či pouhou kolonizaci od invazívního onemocnění. V novější době byla pozornost věnována rozvoji technik k průkazu samotných antigenů. Úspěšné metody byly vyvinuty pro kryptokokózu a histoplasmózu; pro oportunní mykózy, t.j. aspergilózu a kandidózu, však jsou výsledky převážně neuspokojivé. Malá citlivost testů je výsledkem přechodnosti výskytu dokazovaných antigenů v těle hostitele nebo nemožnosti testovat současně větší počet diagnostických antigenů. K překonání těchto problémů byl současný výzkum zaměřen na použití dokonaleji purifikovaných antigenů, monoklonálních protilátek a zjemnění dosavadních nebo zavedení nových citlivých testů. Je podán přehled výhod a nevýhod v současnosti používaných imunodiagnostických metod.

The incidence of systemic fungal diseases has increased significantly over the last decade. During that time considerable research has been done in isolating and characterizing new antigens and developing new technology. However, few new immunodiagnostic tests for the mycoses have come into routine use, due to lack of appropriate evaluations or availability.

Most of the immunological tests currently in use have some limitations and proper interpretation of test results is enhanced if the laboratory has access to information on the patient's clinical history, symptoms, treatment, occupation, history of travel, and residence. In many situations where newly isolated and characterized antigens or their homologous antibodies have been incorporated into tests, the tests have demonstrated inadequate sensitivity. Accordingly, emphasis has been placed on interpreting the clinical relevance of acceptable conventional tests, and on improving the quality of the antigens or antibodies used therein. Although the established tests demonstrate moderate to good sensitivity and specificity, more rapid and accurate tests are needed. To assure their widespread use, such tests would have to be extensively evaluated, standardized, and made commercially available. Use of such tests would lead to early diagnosis of fungal infections and to the prompt delivery of therapy. Furthermore, such tests might allow accurate monitoring of the course of the disease and the effects of therapy.

Most fungal immunodiagnostic tests are designed to detect antibodies to specific pathogens. These tests though far from optimal have proven useful for diagnosing mycotic infections mainly in the immunocompetent host. Some, however, exhibit cross-reactivity, and fail to distinguish active from past infection, and colonization from invasive disease. The development of assays specific for antibodies associated with early active infection are needed. More recently, researchers have direct their efforts to the development of antigen detection procedures. Whereas such procedures have been successfully developed for cryptococcosis and histoplasmosis, those for the opportunistic mycoses, i.e. aspergillosis and candidiasis have been generally unsatisfactory (de Repentigny and Reiss 1984). The main problem is insensitivity, apparently resulting from the transient nature of the targeted antigen(s).To overcome the aforementioned problems, current immunologic research has focused on the use of more purified antigens, monoclonal adsorbed polyclonal antibodies, and the refinement or introduction of more sensitive assays. The purpose of this presentation is to present an overwiev of the immunodiagnostic tests currently used, and to target any obvious shortcomings.

Aspergillosis

Invasive aspergillosis is difficult to diagnose ante mortem. It is however, being diagnosed post mortem with increasing frequency in immunocompromised patients. Specific antibodies to *Aspergillus* spp. can readily be detected in the serum of the patients with non-invasive aspergillosis.

Precipitin and counterimmunoelectrophoresis (CIE) tests have proven to be practical and reliable. A review of the literature indicates that the percent of antibody-positive immunosuppressed aspergillosis cases detected by ID varied from 0 to 70% (Kaufman 1983), and that sensitivity could be increased to 79-90%, through the use of radio- and enzyme- immunoassays (Kaufman 1983). For improved sensitivity a battery of *Aspergillus* spp. antigens, i.e. *A. fumigatus, A. flavus,* and *A. niger* should be used in enzyme immunoassay (EIA) and immunodiffusion (ID) tests. Antibody tests have demonstrated limited diagnostic value and have not replaced biopsy as the definitive means to establish an antemortem diagnosis of invasive disease. The fact, however, that *Aspergillus* spp. antibodies can be detected in sera from immunocompromised patients suggests that development of more sensitive assays for unique antibodies could lead to increased diagnosis of invasive aspergillosis.

Radio- and enzyme-immunoassays have been used with moderate success for the detection of aspergillosis antigenemia, but none have gained universal acceptance. A competitive binding radioimmunoassay (RIA) for galactomannan is potentially useful for detecting *Aspergillus* spp. antigenemia. The method has a sensitivity of 74% and a specificity of 90% (Talbot et al. 1987). However, since antigenemia is not always evident, frequent monitoring of granulocytopenic patients is important. A latex agglutination (LA) test with a sensitivity of 93% for detecting serum galactomannan in invasive aspergillosis has received mixed reviews and awaits further evaluation (Dupont et al. 1990). An EIA inhibition test for aspergillosis antigenemia and antigenuria has been developed using either a polyclonal or monoclonal antibody to galactomannan antigen (Rogers et al. 1990).The test reportedly provides 95% or greater sensitivity, specificity, and predictive values for invasive disease. Serial specimens, however, had to be tested to achieve such values.

As indicated earlier histologic studies are important in diagnosing invasive aspergillosis. The histologic recognition of an *Aspergillus* sp. in biopsy specimens, however, can be difficult, because *Fusarium* spp., *Pseudallescheria boydi*, and other hyaline opportunistic fungi are morphologically similar. Specific antibodies are needed to enable an unequivocal identification of Aspergillus spp. in tissue (Kaufman 1992a).

Blastomycosis

In recent years serologic tests for the diagnosis of blastomycosis have improved substantially as a result of increased purification of the *Blastomyces dermatitidis* A antigen. Complement fixation (CF) and ID tests are specific but demonstrate poor to moderate sensitivity, ranging from 40 – 65% (Kaufman 1992b). Increased sensitivity ranging from 80 – 88% accompanied by excellent specificity was ac-

complished through development of EIA, western blot and RIA tests. Cross-reactions, when apparent, are mainly with histoplasmosis case sera (Kaufman 1992b). Recently Klein and Jones (1990) described a RIA for antibody to 120-kDa cell wall protein of *B. dermatitidis*. This assay using the 120-kDa antigen which is identical or very similar to the A antigen, demonstrates a sensitivity of 85 % and total specificity when sera are diluted above 1:40. The aforementioned tests appear very promising and await further evaluation.

Candidiasis

Candidiasis is the most common life-threatening systemic mycotic infection. Non-culture methods are needed to complement culture techniques. Current immunodiagnostic methods suffer from problems with sensitivity and or specificity. Immunodiffusion and counterelectrophoretic antibody detection methods while demonstrating reasonable sensitivity frequently do not allow distinction between infected and colonized cases. A variety of antigen assays have been developed but none have been accepted for widespread use. The Cand-Tec latex agglutination test for an uncharacterized heat-labile antigen has a sensitivity of 55% (Ness et al. 1989). The sandwich mannoprotein EIA is reliable for detecting 65-70% of candidiasis cases among human cancer patients (Meckstroth et al. 19). The poor to moderate sensitivity exhibited by both tests does not provide an optimum negative predictive value for diagnosing invasive candidiasis.

Candida-enolase a 48 kDa cytoplasmic antigen, has also proven useful as a diagnostic marker of candidiasis in neutropenic patients. The test demonstrates a sensitivity of 85% and a specificity of 96% when multiple serum specimen are studied, and a sensitivity of approximately 54% when only single specimens are studied (Walsh et al. 1991). The test is poor with non-granulocytopenic patients. To achieve the improved sensitivity and specificity it may be best to perform tests for both antigens and antibodies.

Cryptococcosis

A variety of diagnostically and prognostically useful tests for cryptococcosis have been developed during the last two decades. These procedures are an indirect fluorescent antibody (IFA) technique, and a tube agglutination (TA) test for cryptococcal antibodies, and a LA test and a EIA for cryptococcal antigen. Antibody tests are of value in the detection of early or localized cryptococcosis. They are, however, less specific than the antigen tests (Kaufman and Reiss 1992).

The EIA detects cryptococcal antigen earlier and at lower concentrations than the LA test. It is also not subject to prozone reactions and detects about 6 ng of the capsular polysaccharide per ml, in contrast to 35 ng/ml detected by the LA

test. The sensitivity of the EIA has been further increased as a result of using monoclonal rather then polyclonal antibody in the detection system.

The LA test with serum is limited by the occurrence of false-positive reactions by RF as well as false negative reactions by soluble immune complexes, problems which can be eliminated by treatment with pronase (Kaufman and Reiss 1992). Inspite of these problems, LA assays for cryptococcal antigen have proven invaluable in the diagnosis of chronic meningitis. Antigen has been detected in the CSF of over 90% patients with such disease. The test, however, is less sensitive for detecting non-meningeal cryptococcosis. Because the LA test with pretreated specimens is rapid, very specific, diagnostically and prognostically valuable, and simple to perform, it remains the most widely used procedure for detecting cryptococcal antigen. At present, effective diagnosis of cryptococcosis is best achieved through the concurrent use of antigen and antibody tests.

Histoplasmosis

Serologic evidence is often the prime factor in the definitive diagnosis of histoplasmosis. Such evidence may be obtained through commercially available CF, ID, and LA antibody tests, used singly or in some combination, or by the double-antibody sandwich RIA for *H. capsulatum* antigen (Kaufman 1992c). With the currently available yeast-form and histoplasmin antigens, the CF test, although sensitive, is not entirely specific. The *H. capsulatum* yeast-form antigen in particular, may cross-react with sera from patients with blastomycosis, coccidioidomycosis, and other mycoses.

The histoplasmosis ID and CF tests, with histoplasmin as antigen, will react with about 80% of serum specimens from patients with histoplasmosis. Because the histoplasmin H and M antigens are specific for *H. capsulatum*, the ID test provides a more accurate diagnosis with sera that have low titres or cross-react in CF tests (Kaufman 1992c).

The histoplasmin LA test although satisfactory for detection of early acute primary infection, yields negative results with sera from many persons with chronic histoplasmosis.

Detection of *H. capsulatum* polysaccharide antigen provides a rapid means of diagnosing disseminated histoplasmosis in persons with AIDS, in those otherwise immunosuppressed, and in non-immunocompromised patients. The HPA test is particularly important in testing AIDS patients residing in the histoplasmosis endemic areas, since up to 20% of them develop disseminated histoplasmosis. The antigen is found in the urine of 90% and in the blood of 50% of patients with disseminated histoplasmosis. However, 50 to 75% of the patients with less severe disease (non-disseminated) may be negative for antigenuria (Kaufman 1992c). Antigenuria detection offers an opportunity for early diagnosis of histoplasmosis

since it is usually present at the time of clinical manifestations, whereas cultures may not become positive until 2-4 weeks later. The RIA test is not without limitations. Prior to disseminating disease, tests for *H. capsulatum* polysaccharide may be negative. The test is also not entirely specific, false-positive results have been reported with urine or serum samples from patients with disseminated blastomycosis, paracoccidioidomycosis, and a patients with coccidioidal meningitis. Furthermore, the test is complex, reagents and kits are not yet commercially available, and for the present testing is performed at only one laboratory. Obviously a non-isotopic variation of the method would contribute to its more extensive use.

Pythiosis

Pythiosis is a disease of animals and humans caused by *Pythium insidiosum*. The clinical symptoms of the disease are not pathognomonic and diagnosis is based upon isolation and identification of the etiologic agent. Unfortunately, the etiologic agent is not always successfully cultured and histopathologic studies do not always allow a definite diagnosis. The serodiagnosis of pythiosis could circumvent the need for extensive, time-consuming, costly cultural and histopathological studies in humans and animals with suspected pythiosis. Preliminary studies indicate that the ID test with culture filtrate antigens of *P. insidiosum* specifically diagnoses the disease in animals. Todate a decline in precipitins suggests successful immunotherapy or surgery (Mendoza et al. 1986).

Specific FA reagents may be used to identify this fungus-like organism in histopathologic sections (Mendoza et al. 1987).

Paracoccidioidomycosis

CF, EIA, ID, and CIE tests are useful in the diagnosis of paracoccidioidomycosis and for monitoring responses to treatment. The ID and CIE are simple to perform and are among the most specific procedures. CF, EIA, and other tests using nonpurified antigens are sensitive but less specific.

The CF test will detect antibodies in 79 to 96% of patients with paracoccidioidomycosis (Kaufman and Reiss 1992). However, the CF results with filtrate antigens prepared from multiple or single strains of the yeast form of *Paracoccidioides brasiliensis* are not always specific, and cross-reactions may occur with sera from patients with other diseases, particularly histoplasmosis. It is generally accepted that the ID test is the most practical and specific test for diagnosing paracoccidioidomycosis. With reference sera, it is entirely specific and has a sensitivity of 95% (Kaufman and Reiss 1992). An initial serodiagnosis of paracoccidioidomycosis can be obtained in over 98% of cases with the concomitant use of the ID an CF tests.

A 43 kDA glycoprotein is the major precipitinogen (Puccia et al. 1986) and appears to be identical to the E2 antigen described by Yarzabal et al. (1977) and antigen 1 described earlier by Restrepo and Moncada (1974). The 43 kDa antigen, however, demonstrates cross-reactivity in the EIA and its utility in EIA and other quantitative assays awaits further study. Recently the 43 kDa glycoprotein was demonstrated by the immunoblot technique (Mendes-Giannini et al. 1989) in the sera of patients with paracoccidioidomycosis. The diagnostic and prognostic value of this and other *P. brasiliensis* antigens in antigenemia or antigenuria tests require further study.

In the absence of multiple budding yeasts forms the histological characteristics of *P. brasiliensis* may not be specific and the fungal elements may be confused with those of *B. dermatitidis, C. immitis* and *H. capsulatum.* Polyclonal FA reagents though specific must be adsorbed. Preliminary studies (Figureoa et al. 1994) suggest that *P. brasiliensis* can be specifically immunohistologically identified with monoclonal antibodies directed against a 22- to 25- kDa cytoplasmic antigen. Additional studies with blastomycosis, coccidioidomycosis and histoplasmosis case tissues are needed to confirm the specificity of these antibodies.

Zygomycosis

Zygomycetes are the third leading cause of fungal infection in patients with hematologic malignancies. Greater than 90% of disseminated zygomycosis cases were diagnosed post mortem. Early diagnosis may lead to greater survival and effective treatment. Development of sensitive and specific immunologic tests could provide rapid and noninvasive diagnostic tools.

A 2 hr. EIA using homogenate antigens of *Rhizopus arrhizus* and *Rhizomucor pusillus* was evaluated against an ID test using the *R. arrhizus* antigen (Kaufman et al. 1989). In tests with 43 proven zygomycosis case sera, the sensitivity of the EIA was 81% and the ID 66%. The specificity of the EIA was 940 whereas that of the ID test was 91%. The sensitivity of these tests are far from optimal and non-specific reactivity was particularly evident with sera from patients with aspergillosis and candidiasis. Interestingly, the tests are capable of detecting antibodies in cases of systemic zygomycosis caused by species other than *Rhizopus arrhizus* and *Rhizomucor pusillus.* The EIA provides advantages over the ID test, which requires serum to be concentrated and preincubated for 3 h before the addition of antigen. The EIA has potentials, but obviously, additional studies are needed to improve its sensitivity and specificity (Kaufman et al. 1989). Toward this goal Kappe et al., (Kappe et al. 1994) are attempting to identify the immunodominant antigens in zygomycosis. They have reported that 24- and 32 kDa antigens found among several members of the class of Zygomycetes appear promising for serodiagnosing zygomycosis.

CONCLUSIONS

Studies to identify and characterize new antigens coupled with new technology are necessary to readily detect non-transient diagnostic antigens associated with systemic mycotic infections in neutropenic patients. Once these new methods are developed, extensively evaluated and standardized, their positive impact on diagnosis will be furthered by their being made commercially available and thus widely used.

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