Developments in the serological diagnosis of opportunistic fungal infections

J. P. Burnie

Department of Medical Microbiology, University of Manchester, Manchester M13 9PT, UK

Recent years have seen some progress in the design of serodiagnostic tests for the medically important invasive mycoses. This has included the introduction of latex agglutination tests for antigen detection in systemic candidosis and invasive aspergillosis. The circulating cytoplasmic antigens of Candida albicans and Aspergillus fumigatus have recently been delineated as heat-shock proteins of approximate molecular weight 90 KD.

Introduction

The incidence of systemic fungal infection has markedly increased in recent years. This reflects a rise in the number of patients with predisposing conditions such as neoplasia, acquired immune deficiency syndrome (AIDS), major surgery, antibiotic therapy, corticosteroids and cytotoxic therapy, and intravenous and urinary catheterization. The serodiagnosis of these infections has become increasingly important. It can be split into two main areas, the detection of serum antibody and the detection of serum antigen. Both of these present problems.

There may be no antibody production despite disseminated fungal infection. Antibody production may occur so late on in the disease that it is of prognostic rather than diagnostic value. This is the case in disseminated candidosis (Matthews, Burnie & Tabaqchali, 1984). Antibody may also be present in the control population making it difficult to distinguish between these and infected patients (Matthews et al., 1986).

The detection of serum antigen also poses problems. It must be associated with infection rather than colonization. It must be present sufficiently early for antifungal therapy to be started with some chance of success. The antigen must be conserved within the species and specific to that species. It must not cross-react with other human or bacterial antigens. The antigen must be detectable by antibody. It must be possible to raise either polyclonal or monoclonal antibody against the circulating antigen able to detect it in sera. The reaction should be quantifiable as this may help to distinguish among background, colonization and infection. Once this has been obtained a test must be developed that is sufficiently rapid to give results of value to clinicians. It must be easy to perform and not subject to inter-laboratory variation. It must also be possible to supply known positive and negative controls.

This paper will summarise how far these requirements have been achieved in candidosis, invasive aspergillosis, cryptococcosis and some other opportunistic fungal infections.
Systemic candidosis

Antibody detection

The first serological tests for systemic candidosis were based on the detection of specific antibodies with whole cell agglutination, latex agglutination, counter immunoelectrophoresis (CIE), indirect immunofluorescence, passive haemagglutination, radioimmunoassay (RIA) and enzyme immunoassay (EIA) (de Repentigny & Reiss, 1984). These tests lacked sensitivity because it takes time for an antibody response to develop, and immunosuppressed individuals are often unable to mount a good humoral response. Specificity is poor because the presence of antibodies to mannan does not distinguish between colonization and invasive disease nor does it distinguish between recent past infection and active disease. Attempts to increase sensitivity by RIA or EIA are associated with a loss of specificity (Lehman & Reiss, 1980a). Antibodies to cytoplasmic antigens occur far less commonly but are still detectable in 22% of patients who are neither infected nor colonized by Candida albicans (Richardson, Smith & Warnock, 1983). Because the mycelial phase is more characteristic of invasive disease cytoplasmic antigens have been prepared from this but the sensitivity has been reduced (Hopfer & Gröschel, 1979).

Metabolite detection

Assays have been developed for the detection of circulating Candida sugar metabolites, such as mannose and arabinitol. These can be quantified by gas liquid chromatography, but samples have to be derivatized and this limits the number that can be processed in a given day. Arabinitol is cleared by the kidneys so that the serum concentration has to be adjusted in patients with renal insufficiency to give the ratio of arabinitol to creatinine. More recently it has been found that arabinitol and mannose concentrations and the arabinitol/creatinine ratio are higher in high-risk control patients without candidosis than in normal blood donors; this greatly reduces the overall sensitivity of the assay system (de Repentigny et al., 1985).

Antigen detection

Assays have been developed for the detection of circulating Candida antigens. Serum mannan has been detected by CIE, haemagglutination inhibition, EIA and reverse passive latex agglutination (RPLA) (Warren, Richardson & White, 1979; Kerkering, Espinel-Ingroff & Shadomy, 1979; Weiner & Coats-Stephen, 1979; Meunier-Carpentier & Armstrong, 1981; Kahn & Jones, 1986). Mannan circulates at very low concentrations in the form of soluble immune complexes which have to be dissociated before it can be reliably detected. Even with this dissociation step only 70% of patients with systemic candidosis gave positive results with either EIA or RIA (Warren et al., 1979; Lehman & Reiss, 1980b).

Several assay systems have been developed for the detection of the circulating cytoplasmic antigens of C. albicans. Araj et al. (1982) developed an EIA for the quantitation of cytoplasmic antigen in the sera of cancer patients. They found elevated antigen levels in all seven patients with proven disseminated candidosis, nine of 13 patients with suspected systemic candidosis and five of 17 patients with a fever of unknown origin.
The most widely available assay system for detecting candida cytoplasmic antigens has been the RPLA test. Gentry et al. (1983) first described this method and it has subsequently become widely available as the Candtec kit (Ramco Laboratories, Houston, Texas, USA). They found that 30 of 33 systemically infected patients had an antigen titre of 4 or greater, but none of 100 normal controls gave positive results. Two patients with systemic candidosis gave negative results and one showed non-specific agglutination. The antisera used to sensitise the latex particles was raised against heat-killed candida blastospores, but measured a heat-labile antigen. In a subsequent evaluation of the Candtec kit in 30 systemically infected patients it was found that 20 had antigen titres of 4 and 10 had antigen titres of 8 (Burnie & Williams, 1985). One of 81 colonized patients had a titre of > 4 and this patient had a colonized intravenous catheter. Price & Gentry (1986) demonstrated that 13% of rheumatoid-factor-positive patients showed non-specific agglutination with titres of > 2. Applying the Candtec test to 128 patients at high risk of developing systemic candidosis they found 68 patients with titres of ≥ 4 but only 44 were diagnosed as having disseminated candidosis or received amphotericin B. Bailey et al. (1985) compared the Candtec test with another RPLA test in which particles were coated with rabbit antiserum raised against heat-killed \( C. albicans \) blastospores. Both tests produced disappointingly low titres and antigen tended to be detectable only late in the disease. Sera from cases of systemic candidosis were pre-treated with a protease and heated so as to dissociate antigen/antibody complexes. Kahn & Jones (1986) compared the Candtec test with a latex test for mannan in which particles were coated with antibody against candida mannan. In 23 episodes of invasive candidosis in leukaemic patients, antigenaemia was detected in 18 cases (78%) by the test detecting mannan and 11 (48%) by Candtec.

Fung, Donta & Tilton (1986) examined 83 serum samples from 24 patients infected with \( C. albicans \), six of whom had invasive disease. They reported the Candtec test to have a sensitivity of 45% and specificity of 97% at a titre of > 4 and a sensitivity of 71% and specificity of 98% at a titre of > 8. Ness, Vaughan & Woods (1989) evaluated the test during 217 admissions of 200 patients undergoing intensive chemotherapy or bone marrow transplantation. Eleven patients developed systemic candidosis of whom six gave positive antigen test results. Of the 60 patients who died, 41 underwent autopsy examination and 29 had detectable antigen. The latex test was positive in 30 (20-5%) of the survivors and 10 (53%) of the unautopsied patients. The false positive results were associated with raised serum creatinine concentrations, a finding which was contested (Price & Gentry, 1990).

In 1985 I was involved in designing a RPLA test for the detection of candida antigen. Antibody was raised against an extract of \( C. albicans \) strain NCPF 3153 made with an X press (LKB, Bromma, Sweden) (Burnie, 1985). Latex particles were coated with this hyperimmune serum and evaluated as a diagnostic reagent during the next five years (Burnie, 1985; Burnie & Matthews, 1987; Matthews & Burnie, 1988c, 1988d). Colonized patients clearly gave lower maximum antigen titres than systemically infected cases. High levels of antigen in these patients tended to be associated with intravenous line colonization or candida urinary tract infection. In both these types of patients antifungal therapy may be necessary so that there is an overlap between colonization and infection. The systemically infected patients were subdivided into those who were neutropenic and those with normal white cell counts. There were relatively high maximum antigen titres in the 126 non-neutropenic patients, with a distribution of titres: 4 (2 cases), 8 (5 cases), 10 (50 cases), 20 (46 cases), 40 (20 cases)
and 80 (3 cases). In the 74 neutropenic patients the distribution was as follows: no antigen (5 cases), and titres of 2 (10 cases), 4 (17 cases), 8 (15 cases), 10 (14 cases), 20 (7 cases), 40 (4 cases) and 80 (2 cases). In the individuals with a very high maximum antigen titre (e.g. 80) these were recorded just before death. The difference in the maximum antigen titres between the two groups of patients occurs because the major detectable antigen is a breakdown product.

These results refer to the maximum antigen titres detected and these contrast with those obtained when the diagnosis of disseminated candidosis was first suggested. Only three of six patients with lymphoproliferative disorders had an antigen titre of 8 when first tested. Nevertheless, two further patients subsequently reached this titre once amphotericin B treatment had been started (Matthews & Burnie, 1988c).

The titre altered with the outcome of the patient. After an initial rise when therapy was started it fell rapidly in those patients who responded successfully but remained positive in those patients who subsequently died. A persistent antigen titre of $\geq 4$ carried a bad prognosis. The test was good at detecting infection due to C. parapsilosis where high antigen titres were detected (Matthews & Burnie, 1988c). It was reasonable at detecting infection due to C. tropicalis, C. krusei and C. guilliermondii. It missed infection due to C. (Torulopsis) glabrata (Matthews et al., 1990).

It must be remembered that RPLA methods need positive controls to ensure that the test is working, negative controls to ensure that the latex is not spontaneously agglutinating and a serum control for non-specific reactions.

The 47KD antigen of C. albicans

Serial serum samples from 92 patients with proven disseminated candidosis were examined by immuno-blotting. Of the 74 patients with a detectable antibody response, 92% had antibody against a 47KD component of C. albicans, 40% had antibody to a 60KD band and the remaining 24 bands were only recognised by a minority of patients (Matthews et al., 1984, 1987).

Immunodominant antigens of similar molecular weights to the 47KD band have been described by others in serum and urine (Greenfield & Jones, 1981; Strockbine et al., 1984; Au-Young, Troy & Goldstein, 1985; Neale, Muir & Drake, 1987; Ferreira et al., 1990). All survivors (30% of patients) were found to have a major antibody response to the 47KD antigen whereas fatal cases produced little or no antibody or initially had antibody which faded as their condition deteriorated (Matthews et al., 1984, 1987). Neutropenic patients as a group differed from non-haematological patients in that 45% produced a mainly IgM response, failing to sero-convert to IgG. Nevertheless, the survival rate amongst patients producing IgM was similar to that in non-neutropenic patients who produced IgG. Antibody was also found in patients with chronic mucocutaneous candidosis (Burford-Mason, Matthews & Williams, 1987) and AIDS (Matthews et al., 1988b).

The 47KD antigen was isolated from the serum and urine of patients by affinity chromatography (Matthews et al., 1987; Ferreira et al., 1990). The antigen was specific to the species C. albicans and conserved within that species as demonstrated by peptide mapping (Matthews, Wells & Burnie, 1988e). A dot immunobinding assay, based on affinity purified antibody against this antigen, was capable of detecting circulating antigen in the serum of patients. The rate of detection of systemic candidosis in neutropenic patients was 77%, compared with 55% when a total antibody probe was
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used and 29% for the RPLA. All three assay systems were positive in over 73% of infected patients who were not neutropenic (Matthews & Burnie, 1988a).

The identity of the 47KD antigen was established by cloning it into lambda gt 11 (Matthews & Burnie, 1989). The library was screened with rabbit hyperimmune sera and sera from patients with AIDS. The positive clones were sequenced. Their amino-acid sequence revealed an 83% homology with a heat shock protein (HSP) 90 of Saccharomyces cerevisiae. Homologous HSPs have been found in man where there are two separate genes encoding for a heat inducible and a constitutively expressed HSP90 (Hickey et al., 1986). A monoclonal has been prepared against the HSP90 of C. albicans and this has shown that the 47KD antigen is expressed at 23°, 30° and 37°C. At 30° and 37°C the monoclonal detected a higher molecular weight antigen of apparent molecular weight 92KD. The antigen is conserved within all the isolates of C. albicans examined and it is present in both the yeast and the mycelial form.

The 48KD antigen of C. albicans was also characterized by immuno-blotting human sera (Strockbine et al., 1984). It was thought to be the same antigen as the 47KD antigen of Matthews et al (1984). However, direct amino-acid sequencing of this antigen has identified it as an enolase. A monoclonal against this antigen has been evaluated as a diagnostic reagent (Walsh et al., 1989); a liposome immunoassay employing this monoclonal is now being marketed (Directigen, Becton Dickinson, Cockeysville, Maryland, USA).

Invasive aspergillosis

Invasive aspergillosis is second only to candidosis as a cause of fungal infection in cancer patients (Young et al., 1971). There has been a marked increase in the incidence and subsequent mortality in recent years (Fraser et al., 1979). The commonest clinical presentation is that of unremitting fever and the development of pulmonary infiltrates despite broad-spectrum antibiotic therapy (Meyer et al., 1973). Pulmonary involvement occurs in about 93% of cases and most commonly takes the form of a broncho-pneumonia or haemorrhagic infarction (Fisher et al., 1981). The latter is due to vascular invasion and may mimic a pulmonary embolus with infarction, pleuritic pain, fever and tachycardia. The culture of aspergillus from respiratory secretions is not a reliable means of confirming the diagnosis because this may reflect only colonization (Fisher et al., 1981). The definitive diagnosis depends on the demonstration of the characteristic hyphae in tissue sections, but patients may be too ill for these invasive investigations to be undertaken. Serodiagnosis has been performed by antibody and antigen detection.

Antibody detection

Serological tests such as double diffusion and CIE are of value in the diagnosis of aspergillosis and allergic broncho-pulmonary aspergillosis (Dee, 1975; Warnock, 1977; Mackenzie, Philpot & Procter, 1980). This contrasts with the poor results in invasive aspergillosis (Young & Bennett, 1971; Schaefer, Yu & Armstrong, 1976). Other more sensitive antibody detection systems are equally poor in diagnosing invasive disease (Schonheyder, 1987).

Immunoblotting has also been used. Antibody was detected against nine components of the fungus ranging in molecular weight from 88-33KD. It was present
against a 40KD component in most patients with invasive disease and absent in ten sera from healthy controls (Matthews et al., 1985). This work was extended to a further 20 cases where antibody, although specific to invasive disease, was detectable in only 20% of cases (Burnie & Matthews, 1989).

**Antigen detection**

Recent years have seen an interest in antigen detection in invasive aspergillosis. Encouraging results were obtained by Reiss & Lehmann (1979) who demonstrated that galactomannan extracted from the mycelium of *Aspergillus fumigatus* with cold dilute alkali reacted with antiserum specific to an antigen that circulated in invasive aspergillosis both in rabbits and in man. The galactomannan was purified by affinity chromatography and had a molecular weight of between 25 and 75KD. Monoclonals raised against galactomannan are now being used as the basis of a commercial RPLA test for aspergillus antigen detection (Pastorex Aspergillus, Diagnostics Pasteur, Marnes-la-Coquette, France).

In an animal model it was possible to demonstrate galactomannan in the serum of four of 12 rabbits lethally infected with *A. fumigatus*. The concentration ranged from 108 to 356 ng/ml (Dupont et al., 1987). The results of assaying the urine were far more encouraging in that it was possible to demonstrate galactomannan in all the rabbits, with concentrations varying from 24 to 1900 ng/ml. The urine from seven of 13 patients with invasive aspergillosis had galactomannan concentrations ranging from 1 to 83 ng/ml. The level of antigen correlated with the extent of the disease. An inhibition EIA detected 10 ng/ml of antigen in the serum of infected rabbits (Sabetta, Miniter & Andriole, 1985). A competitive inhibition radio-labelled antigen binding assay has also been developed and applied to 616 sera from 79 haematology patients admitted on 152 occasions (Weiner et al., 1983; Talbot et al., 1987). Invasive aspergillosis developed during 24 admissions in 22 of the patients. The maximum antigenic activity was significantly higher in patients with invasive aspergillosis than in controls. The level of antigenic activity selected as the cut-off value gave a sensitivity for the assay of 74% and specificity of 90%.

The problems with designing a test for detecting antigen in invasive aspergillosis stem from the difficulties in defining which antigen to detect. Recently, immunoblot fingerprinting (Burnie et al. 1989) has indicated that there is considerable antigenic heterogeneity between isolates. This is compounded by the fact that individual infections may be due to more than one strain of *A. fumigatus*. There also have been problems with the more complex serodiagnostic techniques, with inter-laboratory variation. Attempts to diagnose invasive aspergillosis by latex agglutination have had limited success. At a diagnostic cut-off titre of 8 it was possible to diagnose only five of 20 proven cases of systemic aspergillosis (Burnie & Matthews, 1989). Nineteen of the 20 cases died and when a serum taken just before death was examined, a titre of 8 was reached in 12 cases. The latex test was negative in four patients in whom the sputum was colonized with *A. fumigatus* in the absence of invasive aspergillosis.

Attempts to improve the serodiagnosis have led to the identification of potential circulating aspergillus antigens. Phillips & Radigan (1989) developed a rabbit model of invasive aspergillosis and purified the circulating antigen by affinity chromatography. They demonstrated an 80KD antigen but failed to detect it in the serum of three human cases of invasive aspergillosis. Yu, Niki & Armstrong (1990) developed a rat model and...
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by examining urine directly with SDS-PAGE and immunoblotting showed antigenic bands at 88, 40, 27 and 20KD. The antibody which formed the basis of the latex agglutination test has been used to extract circulating antigen from the sera of patients (Burnie & Matthews, 1989). This detected an 88KD band and cross-reacted with a monoclonal raised against the HSP90 of C. albicans. There may be a circulating antigen analogous to the HSP90 of C. albicans in invasive aspergillosis.

Cryptococcosis

Cryptococcus neoformans is an important pathogen causing fatal meningitis and disseminated cryptoccocosis in normal and immuno-compromised hosts. It is especially important in patients with AIDS. The major serological test for identifying patients with cryptococcal infection is latex agglutination. This test is based on latex particles coated with antibody raised against the cryptococcal capsule (Bloomfield, Gordon & Elmendorf, 1963). There are at least four serotypes A, B, C and D. Serotype A is the most important in clinical infection (Tanphaichitra, Srimuang & Sahaphong, 1989). The RPLA test reliably detects antigen in CSF but a positive serum result may reflect non-specific agglutination of the latex particles (Armstrong, 1989). This can be detected by performing a test for rheumatoid factor. In addition patients who are infected by Trichosporon species (which possess a cross-reacting antigen) may also give a false-positive reaction (McManus & Jones, 1985). The level of antigen is not only helpful in diagnosis but closely follows the response to therapy.

Trichosporonosis

Systemic infection with Trichosporon beigeli (formerly Trichosporon cutaneum) or Blastoschizomyces capitatus (formerly Trichosporon capitatum) is rare but increasingly important. This is partly due to the fact that T. beigeli is resistant to amphotericin B (Walsh et al., 1990). Serodiagnosis has received some attention because patients with trichosporonosis may give a positive reaction in the cryptococcal RPLA test (McManus & Jones, 1985). Matthews et al. (1986) demonstrated that both patients with trichosporon infection and controls had high levels of antibody as measured by immunoblotting against T. beigeli NCPF3077.

Conclusion

This review has outlined the progress that has been made in the serodiagnosis of those opportunistic fungal infections of importance in immuno-compromised patients. The most reliable serodiagnostic test is the RPLA test for the diagnosis of cryptococcal infection; by contrast, there are no reliable tests for invasive mucormycosis. In the case of invasive candidosis recent work has defined a 47KD HSP and the role of this in diagnosis is currently under investigation. In invasive aspergillosis attempts to identify the key circulating antigens have been centered on galactomannan and an 80KD complex.
References


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