The application of monoclonal antibodies to the diagnosis of disseminated mycoses

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Summary

Monoclonal antibodies (MoAbs) have had a major impact on many areas of biomedical research and almost since their advent have been used in the characterisation and identification of diagnostically important antigens of fungal pathogens. Their main significance lies in three, often inter-related areas: a) the definition and characterisation of antigens for use in detection of antibody responses, b) their direct use in the detection of diagnostically useful antigen in body fluids c) their application in immunohistochemical diagnosis. The degree to which MoAbs have been applied varies between fungal pathogens, and they have now been used, for example, in the serodiagnosis of Aspergillus sp., Cryptococcus neoformans, Histoplasma capsulatum and Paracoccidioides brasiliensis. Their use in producing diagnostic tests for other fungi such as Sporothrix schenckii and Penicillium marneffei has been more restricted but considerable potential exists for further development.

Key words

Diagnosis, monoclonal antibodies, fungal pathogens

Generally speaking the gold standards for the diagnosis of disseminated fungal infections are cultural isolation and positive histochemical identification. However serological detection of fungal infections is often of great importance, both at the point of diagnosis and during the monitoring of patient’s response to therapy. This is of particular importance in situations where cultural procedures fail, or where histochemical identification proves to have low sensitivity. Immunohistochemical identification can provide a useful adjunct to the latter, and it has the capability to increase both sensitivity and specificity.

Monoclonal antibodies (MoAbs) are potentially of use in both serodiagnosis (in the definition, characterisation and purification of antigen for use in antibody detection) and in the direct development of antigen detection assays) and in immunohistochemistry. To a degree MoAbs have tended to replace existing polyclonal sera in diagnostic tests, a development which has arisen from the appreciation of the problems inherent in the use of polyclonal antisera, namely: a) batch variation between serum samples b) availability of limited stocks of sera, c) lack of standardisation between various antibody batches, with concurrent inability to compare the results of detection experiments, d) the relative lack of sensitivity of the antisera, e) difficulties in identifying the actual antigen being detected, f) the potential lack of specificity of such reagents, necessitating cross-absorption. In contrast the supply of any given MoAb is effectively unlimited, and...
each MoAb possesses defined chemical reproducibility and specificity. In addition, potentially such reagents will have greater sensitivity due to lower attendant background values, and their production does not require access to a continuous supply of antigen.

That is not to say that the use of MoAbs is problem free, and there are potential pitfalls in the application of these reagents due to intrinsic cross reactivity resulting from the presence of related chemical structures or shared determinants. Other problems may occur as a result of low antibody affinity, and also arise due to low sensitivity resulting from a narrow range of reactivity. It will become evident during the course of this article that despite such potential limitations MoAbs have had a significant impact on the diagnosis of mycoses, and recent developments, particularly with regard to *Histoplasma capsulatum* and *Paracoccidioides brasiliensis* in particular suggest that the application of these reagents still has a great deal to offer.

**Aspergillus sp.** The definitive diagnosis of aspergillosis takes into account data from clinical, radiological, serological and histopathological sources, and it is in regard to the last two areas that MoAbs have a role to play. It should be noted, however that with the exception of the invasive form of the disease, the diagnosis of aspergillosis is not generally difficult. In the case of invasive disease it is of particular concern that diagnosis is made without delay, since prognosis rapidly worsens in the absence of recognition and effective treatment.

A great deal of effort has gone into the detection of specific *Aspergillus fumigatus* antibodies in patients with allergic aspergillosis and aspergillosis [1-3] and MoAbs have played a role in the characterisation of the immunoreactive antibodies which are responsible for such allergic responses. Although probably less useful diagnostically, the detection of antibody responses in individuals with invasive aspergillosis has also been attempted, and MoAbs have also played a useful role in antigen characterisation in this area [4,5].

However, in the context of invasive aspergillosis, and the patient groups who develop this form of the disease, the detection of antigen is conceptually a more effective means of diagnosis than antibody detection can ever be. Most interest has centered around the detection of galactomannan (GM) [6,7]. Until relatively recently the antibody detection methods used were either polyclonal rabbit or human anti-Aspergillus antibodies [8-11]. However MoAbs have now been successfully used in the detection of GM, and they form the basis of commercially available kits. For example the Sanofi-Diagnostic Pasteur kit (France) relies on MoAbs directed against a tetra β(1-3) galactofuranoside [12], which appears to be the immunodominant epitope in GM. Various test formats have been used including latex agglutination [13,14], inhibition ELISA [11] and sandwich ELISA [15]; the latter offers the greatest sensitivity [7,16], with detectable GM levels of 0.5 to 1.0 ng/ml of sero [15]. Overall the sensitivity of the sandwich ELISA appears to be between 67-100%, with some patients having detectable GM in serum even before clinical indications of Aspergillus infection [15-18]. There is some limited evidence that the clinical outcome of disease correlates with the levels of detectable GM [17,19] and the ELISA can also be used with bronchialveolar lavage samples [7,20]. It is noteworthy, however, that the generation of false positive results may still be a problem when detecting GM, with a rate of up to 15% reported recently [21]. Part of this problem may be due to a genuine cross-reaction with other fungi (particularly members of the genus *Penicillium* [21]).

The only other Aspergillus antigen which has been shown to be detectable in body fluids via the use of MoAbs is the 18kDa ribonuclease antigen [22,23]. A sandwich ELISA has been developed which combines either a mouse or rat MoAb with a rabbit polyclonal and which claims a sensitivity down to 1ng [7]. However, this test does not yet appear to have been commercialised.

Immunohistochemical identification of Aspergillus sp. in situ is potentially also a useful tool in the diagnosis of aspergillosis, and several reports deal with the application of MoAbs in this area [24-26]. These studies have typically used MoAbs directed against GM; it is worth noting, however, that in one such report it was concluded that immunoperoxidase staining using anti-GM antibody EB-A1 was no better than conventional histopathology in identifying the presence of infection. There is therefore scope for improvement in this area, possibly by the production of MoAbs which are directed at epitopes other than those present on GM.

**Candida sp.** Invasive candidiasis is by far the most important and prevalent deep-seated fungal infection, and although microscopy and culture are important tools in diagnosis, there has been, and continues to be, considerable scope for the use of methods which rely on serodiagnostic detection. A wide range of techniques have been developed for the detection of anti-Candida antibodies, some of which have been commercialised [27,28]. These tests have some diagnostic value [28-31] and a number of immunodominant antigens have been investigated, including enolase [32-34]. The principal role of MoAbs in this area has been as tools in the definition and characterisation of such antigens [35,36]. However, all systems for the detection of anti-Candida antibodies are dependent on distinguishing between the normal titres found in healthy individuals who are exposed to Candida on a daily basis, and the hopefully elevated titres found in patients with symptomatic disease. There is also the additional problem that the presence of increased levels of antibodies does not necessarily distinguish between life threatening candidiasis and transitory infection which is of only moderate significance, unless the target antigen is one that is expressed solely in deep-seated candidiasis.

Given such circumstances a great deal of interest has focused on the detection of circulating Candida antigen. Mannan was the first of such antigens to be intensively investigated [37,38], and a commercial latex agglutination test (Pastorex Candida assay, Sanofi-Pasteur, France) which utilised a anti-mannan MoAb [39] was subsequently produced. This test is reactive to mannan from most of the commonly occurring species of Candida, with the notable exception of *Candida krusei*. However the sensitivity of the test is not perhaps all that it should be, and a recent study [40] revealed that antigen was detectable in only 25.6% of patients with candidemia. MoAbs have been made against the *Candida albicans* acid proteinase [41] although their use in the detection of this antigen has been handicapped by low sensitivity [42]. In contrast the detection of enolase has proved more effective [40] and the combination of a mouse MoAb with rabbit polyclonal was used as early as 1991 to detect candidiasis in a prospective study in patients with cancer [33]. Finally a MoAb against mannoprotein has also been used to detect antigen in a dot immunobinding format [43], although this approach does not appear to have been taken up commercially.

The Cand-Tec test, which detects a heat labile anti-
gen, has also been extensively employed, although this relies on a polyclonal antibody [44]. The effectiveness of this assay is apparently variable [40, 45] although it may be useful in identifying those individuals likely to develop deep candidiasis [28]. The detection of B- glucan is also potentially diagnostically useful, although tests for this polysaccharide do not utilise MoAbs [46, 47].

It should be evident from the preceding discussion that although a substantial number of antigen detection tests have been developed for candidiasis, all of them have certain limitations, particularly with regard to sensitivity and specificity. Their use in combination has been advocated to compensate for these shortcomings [40]. The impact of MoAbs on these assay systems is perhaps not as great as it might have been, and it would appear that there is still scope for deployment of these reagents in this field.

Polyclonal antibodies have proved of some use in the immunohistochemical diagnosis of candidiasis [48] and MoAbs have also been shown to have some potential in this area [49-51]. MoAb 1B12 [52] appears to be of particular value, with its reported ability to recognise Candida in paraffin-embedded tissue and its lack of reactivity to other fungi. In our experience, a sizeable percentage of MoAbs will not retain reactivity against fungi in paraffin embedded tissue due to antigenic denaturation during processing. Indeed this constitutes perhaps the biggest drawback to the application of MoAbs to this area and it is generally the exception, rather than the rule, to find MoAbs that are as reactive against paraffin embedded material as they are against frozen, lightly fixed material. Finally, it is of note that the application of MoAbs to the identification of Candida has also been extended to the direct and rapid identification of yeast colonies, with a commercial kit (the Bicho-latex albicans test, Fumouze, France) providing a useful tool [53, 54]. This type of application for specific MoAbs has not generally been exploited in the identification of other medically important fungi, which is perhaps surprising given the potential time saving that might be made.

**Cryptococcus neoformans.** Historically the identification of C. neoformans infections has relied on a combination of culture of CSF or the demonstration of encapsulated yeast cells in India ink preparations of CSF. However, the detection of circulating cryptococcal antigen in body fluids has now become probably the most effective and sensitive means of diagnosis [55-57]. Initially the antigen detection system relied on a latex agglutination system in which polyclonal antibodies directed against capsular components were used to coat latex particles [58, 59]. This system generally proved itself to be both sensitive and specific, although there were some reports of problems associated with false positivity [57, 60]. Subsequently the generation of MoAbs against capsular polysaccharide [61, 62] offered scope for the development of tests that did not rely on polyclonal antibodies, and considerable effort has been expended in this area. The existing latex agglutination format has been modified to include MoAbs, with several commercial systems being produced in the early 1990s. These include the Pastorex Cryptococcus test (Sanofi-Pasteur), which when compared to agglutination tests which incorporated polyclonal antibodies, was found to be similarly sensitive and effective [63] and the CRYPTO-LEX (Trinity Laboratories, Inc., USA) [64], which was also found to be highly effective.

ELISA based systems are potentially more sensitive than those using latex agglutination and a number of variations have been developed. These include a two-site non-competitive ELISA that utilises the combination of a polyclonal antibody for antigen capture with a glucuronoxylomannan (GXM) binding MoAb [65] for antigen detection (the PREMIER Cryptococcal Antigen assay, Meridian Diagnostics, Inc., USA [66]). An experimental sandwich ELISA using combinations of IgG and IgM MoAbs [67] has also been used to define the importance of antibody isotype in the effectiveness of antigen capture.

MoAbs have also been produced against non capsule components [68] and one of these (the 115kDa glycoprotein) has been shown to elicit an antibody response [69]. However, antibody detection as a means of diagnosing C. neoformans infections comes in a poor second to antigen detection, and developments in this area have been restricted. It is of note that although some of these non-capsular reactive MoAbs [68] were able to label yeast cells by immunofluorescence antibody test (IFAT) on methanol fixed cryostat sections none of them has subsequently proved useful in identifying yeasts in paraffin wax embedded biopsy material. Indeed there remains a need for a C. neoformans specific MoAb for use in such situations, although the microscopic identification of C. neoformans in tissue is not as problematic as that of other fungal pathogens, largely because of its highly visible capsule. However, the application of polyclonal antibodies in this area [48, 70] demonstrates that there is at least some potential in immunohistochemical diagnosis of C. neoformans.

**Blastomyces dermatitidis.** Serodiagnosis of B. dermatitidis has historically relied on the detection of antibody responses against various antigens, by a variety of techniques [71, 72] including a commercialised enzyme immunoassay [73]. Bradsher [74, 75] has described the serodiagnosis of blastomycosis via antibody detection as problematic, largely because of limitations relating to low sensitivity and low specificity, although one of the more recently described radioimmunoassays claims a sensitivity of 85% and specificity approaching 100% [76]. MoAbs have not played a major role in the elucidation of the antigens used in such assays, nor have they been incorporated in the as yet limited attempts at antigen detection [77, 78].

In terms of immunohistochemical diagnosis polyclonal antibodies have been used with some success [48], and those directed against the 120kDa yeast antigen [76] may be broadly applicable in this area. Thus far there are no reports in the literature dealing with the application of MoAbs to the immunohistochemical diagnosis of B. dermatitidis.

**Coccidioides immitis.** A great deal of interest has focused on the detection of humoral responses against C. immitis, and a number of commercial kits have been made available to detect antibodies [28, 79, 80]. Generally antibody responses in C. immitis are a valuable aid both diagnostically and prognostically [81, 82] and recombinant antigens have now been produced for use in a number of antibody detection systems which promise high sensitivity and specificity [83-85]. The major role of MoAbs has come in the characterization and definition of these antigens [86-88].

There have been some attempts to utilise antigen detection assays, which have relied on polyclonal antibodies; such assays have met with mixed success [89-91]. To date MoAbs have not been used in antigen detection, partly, perhaps, because the success of antibody detection methods has negated the development of such technology. Immunohistochemical diagnosis of C. immitis is depen-
Sporothrix schenckii. Diagnosis of S. schenckii infection relies principally on clinical presentation combined with direct isolation/culture of the organism [92], although serology, via the detection of antibody responses has a role to play [93,94]. Polyclonal antibodies have also been used in immunohistochemical identification [95,96], with some success. There are few reports dealing with the production of MoAbs against S. schenckii, and those that are available relate to antigen characterisation [97]. There is probably no real demand for the development of MoAb based antigen detection ELISAs of the sort which have been recently developed for H. capsulatum and P. brasiliensis; however S. schenckii specific MoAbs would be useful in immunohistochemical identification of this fungus in clinical samples, and this remains a worthwhile area for study.

Penicillium marneffei. Disseminated P. marneffei infections are relatively easy to diagnose because of the characteristic skin lesions which yield readily identifiable yeast forms [98,99]. The organism is also relatively easy to culture from clinical samples, and the characteristic red pigment produced by the mycelial form is also diagnostically useful [98,99]. However there remain obvious advantages to be gained from the early diagnosis of infection prior to the appearance of dermatological involvement. Research into the serodiagnosis of P. marneffei is still in its relative infancy, although several recent papers have identified a number of potentially useful antigens which may be recognised by patients sera [100-102]. Only one attempt has been made at antigen detection, involving the application of a rabbit polyclonal against fission arthroconidia filtrate [103].

Thus far the only report on the application of MoAbs to the diagnosis of P. marneffei infections relates to MoAb EB-A1, which recognises galactomannan in both P. marneffei and Aspergillus sp. [104]. This antibody has been used in the immunohistochemical identification of P. marneffei in formalin fixed specimens; however its lack of specificity is an obvious problem. A pre-absorbed rabbit polyclonal antibody has proved very useful especially in immunocompromised patients. A radioimmunoassay (RIA) developed in the mid 1980s [115] for the detection of a polysaccharide antigen using a rabbit polyclonal antibody has proved very useful especially in immunocompromised patients [116]. Forjtasek et al. [117] reported an attempt to produce MoAbs which could replace the polyclonal antibody used in the RIA. The two monoclonals produced were able to detect antigen in a small percentage of patients with disseminated histoplasmosis who had positive radioimmunoassays results although the MoAbs were considered unsuitable for replacing the rabbit polyclonal. More recently, an inhibition ELISA using a species specific MoAb has been applied to the detection of a 69-70 kDa circulating antigen. This assay appears to be both sensitive and specific [118]. More studies are needed to evaluate the usefulness of this new test in the follow-up of patients.

Histoplasma capsulatum. The diagnosis of histoplasmosis is based upon a combination of clinical observations and laboratory studies. Definitive diagnosis require growth of the fungus from samples of body fluids or tissues although this procedure may take up to four weeks to provide a positive result. Serological tests are important adjuncts to the diagnosis of histoplasmosis because of the difficulties inherent in detecting the organism by culture or by staining.

The most commonly used tests for the detection of anti-H. capsulatum antibodies are the complement fixation (CF) and immunoprecipitation methods which have been extensively reviewed elsewhere [106,107]. Precipitating antibodies detect two heat-sensitive glycoproteins (the M and H antigens) that are considered to be species specific [107]. Both the H and M antigen have been purified [108,109] although generally attempts to isolate these antigens have been concerned more with their serological activity than with their physicochemical properties. MoAbs have played an important role in this area and have been used as an alternative method of purification because of the difficulty in purifying the M factor to homogeneity by methods based on size and charge. Two MoAbs of two different specificities were produced which permitted the characterisation of the M antigen with respect to its size, charge, and number of reactive components [110]. Other MoAbs have also been produced against the yeast antigen of H. capsulatum [111], which recognised epitopes of 62 and 72 kDa; however these reagents showed differing degrees of cross reactivity and no reports of subsequent attempts to use them in serodiagnosis were made. Hamilton et al. [112] used cyclophosphamide ablation to produce a MoAb with a high degree of species specificity which was able to differentiate between H. capsulatum var capsulatum and H. capsulatum var duboisii [113]. Unfortunately this reagent did not prove effective in the development of a serological test. Finally a MoAb was also raised against a 80kDa heat shock protein (hsp) of H. capsulatum [114]. Although this antigen has been shown to mediate protective immunity in mice it is not useful in serodiagnosis because in common with other heat shock proteins (hsps) it shows high homology with equivalent proteins from other dimorphic fungi.

Antigen detection has played an important role in serodiagnosis particularly since antibody titers may be very low or even absent in immunocompromised patients. A radioimmunoassay (RIA) developed in the mid 1980s [115] for the detection of a polysaccharide antigen using a rabbit polyclonal antibody has proved very useful especially in immunocompromised patients [116]. Forjtasek et al. [117] reported an attempt to produce MoAbs which could replace the polyclonal antibody used in the RIA. The two monoclonals produced were able to detect antigen in a small percentage of patients with disseminated histoplasmosis who had positive radioimmunoassays results although the MoAbs were considered unsuitable for replacing the rabbit polyclonal. More recently, an inhibition ELISA using a species specific MoAb has been applied to the detection of a 69-70 kDa circulating antigen. This assay appears to be both sensitive and specific [118]. More studies are needed to evaluate the usefulness of this new test in the follow-up of patients.

Immunohistochemistry is especially useful for the diagnosis of H. capsulatum infections because small cells of H. capsulatum var capsulatum may easily be confused with Pneumocystis carinii, Candida spp., C. neoformans, P. marneffei and S. schenckii. Polyclonal antibodies have been used although they have some limitations [48]. MoAbs raised against the histoplasmian antigens have been used but have been proved to be ineffective when applied in immunohaemolchemical essays. For example MoAbs against the purified M antigen have been found to intensively stain the yeast cells of both H. capsulatum var. capsulatum and B. dermatitidis [95]. The anti-hsp80 MoAb [114] is also of no use in this context because of cross reactivity. Efforts are currently underway to use the new H1C MoAb [118] in immunohistochemistry.

Paracoccidioides brasiliensis. The definitive diagnosis of paracoccidioidomycosis can only be accomplished by laboratory procedures such as direct examina-
tion, culture and serodiagnosis [119]. To date, no serological method for the diagnosis of paracoccidioidomycosis has been made commercially available, despite the fact that serological procedures are of great value [120]. Many different tests have been employed for antibody detection and immunodiffusion and complement fixation are the most widely employed. Historically the majority of these tests suffered from cross-reactivity. Polyclonal antibodies have been utilised in antigen characterization, and have also been used in antigen detection [120,121]. The first report detailing the production of species specific MoAbs used a modification of the standard MoAb technology [122] although these MoAbs were not subsequently used in serodiagnosis. MoAbs produced against the gp43 antigen, which is considered to be the most diagnostically useful antigen [119], have been applied in an capture enzyme immunoassay for the detection of specific human anti-gp43 immunoglobulin G in patients with paracoccidioidomycosis [123]. Using this assay the reactivities of patient sera were found to be significantly higher than those of control sera. More anti-gp43 MoAbs were produced and used to conduct a detailed study on the subcellular distribution of this antigen by immunoelectron microscopy [124]; these reagents were also used for in vivo studies to modulate laminin-mediated fungal adhesion to epithelial cells and pathogenesis [125], but have not, as yet, been used in serodiagnosis.

Several other P. brasiliensis antigens have now also been identified and characterized to a greater or lesser extent using MoAbs. These include the 58 kDa [126] and 22 kDa glycoproteins [127]. The former was recognized by 81% of immune human sera when tested by Western blot and the latter is of more interest as an immunohistochemical marker. More recently, an inhibition ELISA which utilises a MoAb (P1B) directed against an 87 kDa antigen in sera. The sensitivity of this assay is quite high (80.4%), although important cross reactions were also observed. The same MoAb has also been used in the in-vivo ELISA for the follow up of patients during treatment and results demonstrate that it constitutes an important tool for this purpose [129].

**Concluding remarks.** It should be apparent from the preceding review that the application of MoAbs has substantially added to our ability to diagnose systemic mycoses, particularly in regard to the detection of *Aspergillus*, *Cryptococcus*, *Histoplasma* and *Paracoccidioides* antigens in samples from patients. Antigen detection offers advantages over antibody detection, a factor which has largely been responsible for the growth in interest in this area. That is not to say that antigen detection offers a universal panacea to the serological diagnosis of disseminated mycoses, and substantial problems may be encountered relating to immune complex formation, rapid clearance of antigen and indeed the release of low or very low quantities of antigen. Some of these problems might be overcome by the use of mixtures of specific MoAbs directed against different determinants, an approach that as yet has received little attention.

In terms of the actual production of MoAbs great care should be taken in ensuring that they are made against antigens that are actually serodiagnostically useful. For example there are now a substantial number of reports on the production of MoAbs against fungal antigens, and yet comparatively few of these reagents, at least until relatively recently, have been used to produce useful assays. The impact of MoAbs in terms of immunohistochemical diagnosis has been much less profound, and much remains to be done in this area. Finally note should also be made with regard to the diagnosis of mycoses of the potential of the newer molecular biological based technologies, such as PCR based diagnosis, to augment or supercede serodiagnostic/immunohistochemical approaches. However, as yet the use of such methodologies is not generalized and as such there is still potential for an expansion of the application of MoAbs in the field of mycological diagnosis.

**References**

5. Fratamico PM, Buckley HR. Identification and characterization of an immunodomi-


Hamilton AJ & Gómez BL
124
Rev Iberoam Micol 1998; 15: 118-124


98. Drouhet E. Diagnosis of Penicillium marneffei: a new emerging systemic mycosis in AIDS patients travelling or living in Southeast Asia. Review of cases reported in HIV infected patients during the last 5 years compared to 44 cases of non AIDS patients reported over 20 years. J Med Microbiol 1993;4:195-224.


