CROSS-REACTIVITY OF MONOCLONAL ANTIBODIES AGAINST *ASPERGILLUS VERSICOLOR* AND OTHER FUNGI AND THEIR IMPLICATIONS FOR THE DEVELOPMENT OF ANTIBODY-BASED MONITORING TECHNIQUES FOR FUNGI

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ABSTRACT

In order to improve current culture-based monitoring techniques for *Aspergillus versicolor* and other fungi in general, our laboratory is developing species-specific monoclonal antibody (mAb)-based immunoassay techniques. In this study we attempted to enhance the production of mAbs specific for *A. versicolor* by immunizing mice with pre-formed immune complexes to block epitopes of spores known to be cross-reactive with other fungi. We found that immunization with immune complexes did not enhance the production of *A. versicolor*-specific mAbs. While the antibody reactivity to related *Aspergillus* and *Penicillium* species was reduced, the response to other fungi was enhanced. The patterns of cross-reactivity among the 50 tested fungal species not only demonstrate that phylogenetically distant fungi share common epitopes but also that different fungi express a given cross-reactive epitope in variable amounts on a per spore basis.

INDEX TERMS: Monoclonal antibody, cross-reactivity, ELISA, *Aspergillus versicolor*, monitoring techniques

INTRODUCTION

Many fungi have been associated with a variety of adverse health effects (Fung, Hughson, 2003; Kolstad *et al.* 2002) in indoor environments and, therefore, need to be monitored in order to document exposures and complement rational patient management. Current monitoring techniques are either based on the cultivation of environmental samples or the biochemical detection of marker molecules such as (1-3)-β-D-glucan, ergosterol or extracellular polysaccharides (Burge, 2002;

Pasanen, 2001). While these techniques have been useful on a case-by-case basis, they have failed to establish standardized reference techniques for ecological and exposure monitoring purposes. Some of the main problems associated with these techniques are diagnostic challenges due to differential culturability and relative growth potential of different fungi or the introduction of ambiguities in quantitative results due to the differential expression of marker molecules by different fungi on a per spore or unit of biomass basis.

Our approach for the development of objective monitoring techniques for airborne fungi in indoor environments is based on the detection of fungi with enzyme-linked immunosorbent assays (ELISA) using species-specific monoclonal antibodies (mAbs) or phage display-derived peptide reagents. Although antibody-based techniques are being increasingly used for environmental monitoring of chemical contaminants (van Emon, 2001) and for the presence of fungi in food and feed products (Li *et al.* 2000), their use for the detection of fungi in environmental samples has so far been limited. Two of the major problems associated with the application of immunoassays in fungal research are the extensive cross-reactivity of antibodies with a variety of related and non-related fungi and the expression of shared epitopes at differing amounts by each of the cross-reacting fungi on a per spore basis. The analytical implications of cross-reactive antibodies are such that a given ELISA result cannot be translated into actual spore numbers or biomass units for a particular target fungus. This is because identical ELISA results of two samples may be due to the presence of two entirely different mixtures of cross-reactive fungi which happen to express the same total number of antibody epitopes per sample. In order to address these problems in exposure assessment, we are investigating different methods to improve the specificity of monitoring reagents.

Aspergillus versicolor is not only a commonly occurring fungal species in the indoor air spora (Nielsen, 2003; Shelton *et al.* 2002) but it has also been implicated in a variety of adverse health effects (Hodgson *et al.* 1998). In this study we used *A. versicolor* as a model fungus to examine the potential of immune complex immunization for the production of species-specific mAbs that may be of benefit for the accurate spatial and temporal monitoring of the fungus. During immune complex immunization, mice are immunized with the antigen complexed with pre-formed cross-reactive mAbs. The rationale for this approach is that immune complex immunizations have been reported to shift the immune response toward weakly antigenic sites while at the same time tolerizing against immunodominant epitopes (Benkirane *et al.* 1985; Thalhamer, Freund, 1985). They have also been suggested to increase the diversity and the degree of affinity maturation of the resulting antigen-reactive B cells repertoire (Nie *et al.* 1997). Such a directed shift in the immune

response may increase the chances of isolating species-specific mAbs against highly complex antigens such as fungal spores.

In this study, we immunized mice with immune complexes and produced mAbs against spores of *A. versicolor*. Each mAb was then tested against spores of 16 *Aspergillus* species 14 *Penicillium* species and 20 other fungi commonly found in indoor environments. The reactivity of the five newly produced mAbs was also compared to that of a previously reported mAb that was produced against spores rather than immune complexes.

METHODS

Cultivation of fungi and antigen preparation: Fungi were obtained from the American Type Culture Collection and the National Center for Agricultural Utilization Research or isolated from indoor air samples. Isolated fungi were identified based on spore and culture morphology using standard mycological reference literature. All fungi were grown on malt extract agar (2% dextrose, 0.1% peptone, 2% malt extract, 2% agar; Difco, Becton Dickinson, Sparks, MD) plates for 10 days at room temperature. Spores were collected inside a class II biological safety cabinet from air-dried cultures by applying 1 g of glass beads (0.45 – 0.5 mmØ, Braun-Melsungen, Germany) per Petri dish. The lid was put back in place and the plates were gently shaken back and forth to allow the beads to roll across the spore-covered agar. We found that following this procedure ample spores were attached to the beads. Beads were poured into a 50 ml tube and spores were suspended in either phosphate buffered saline (PBS, 10 mM phosphate buffered saline, 138 mM NaCl, 2.7 mM KCl, pH 7.4; Sigma, St. Louis, MO) for antigen preparation or carbonate coating buffer (60 mM sodium carbonate, 140 mM sodium bicarbonate, pH 9.6) for enzyme-linked immunosorbent assay (ELISA) analysis of mAb reactivities. The elution process was enhanced by briefly shaking the tube a few times. Spores were then separated from the beads by decanting the spore suspension after the beads had settled out.

Production of mAbs: The particulate fraction of homogenized spores of *A. versicolor* was incubated for 1h at 37 °C with tissue culture supernatant of either mAb 2B8 (IgG_3) or a mixture of mAbs 4D2 (IgG_{2a}/IgM) and 2C6 (IgG_{2a}/IgM). These mAbs were previously produced against *Penicillium chrysogenum* and shown to be highly cross-reactive with spores of *A. versicolor* (Schmechel *et al.* 2001). Two groups containing two female BALB/c mice were immunized six times intraperitoneally with one of the two different immune complexes. MAbs were produced according to standard technology (Harlow, Lane, 1988). Hybridomas were screened by solid phase indirect ELISA using spores of *A. versicolor*. Positive cell lines were cloned twice by limiting dilution and aliquots were stored in liquid nitrogen.

Cross-reactivity ELISA: Spores of 50 fungi were each incubated in ELISA wells overnight at room temperature (RT) in carbonate coating buffer, pH 9.6. Spores were coated at a concentration of 100,000 spores per ELISA well except *Alternaria alternata* (30,700 spores/well), *Aspergillus umbrosis* (37,500), *Aureobasidium pullulans* (24,750), *Epicoccum nigrum* (5,750), *Fusarium oxysporum* (57,250), *Geotrichum candidum* (18,000), *Stachybotrys cylindrospora* (25,500) and *Ulocladium chartarum* (37,500) were coated with fewer spores because of poor sporulation. MAb reactivity was determined with a previously described alkaline phosphatase-based indirect ELISA (Schmechel *et al.* 2002).

RESULTS

A total of 12 mAbs were produced. Based on their cross-reactivity patterns these could be grouped into 5 different groups. Figures 1-3 show the reactivities of one characteristic member of each group. MAb 15C8 represents 4 similar mAbs. Two mAbs (10E6 and 10F12) each represent three mAbs and the groups of mAbs 3E3 and 5C6 only contained a single mAb. Each column represents the average of 3 replicate ELISA well optical densities (OD) and each error bar represents the corresponding standard error of the mean. Fungal species are listed on the y-axis and the corresponding ODs which were determined after a substrate incubation time (SIT) of 30 min are shown on the x-axis. No *A. versicolor*-specific mAbs were produced. Figure 1 presents the mAb cross-reactivities for 16 *Aspergillus* and one related *Eurotium* teleomorph species, Figure 2 shows the results for 14 *Penicillium* species and Figure 3 shows the results for 19 other fungi commonly found in indoor environments. In addition, each graph also shows for comparison the cross-reactivities of mAb 14D6 which was previously produced against spores rather than immune complexes of *A. versicolor* (Schmechel *et al.* 2002).

The cross-reactivity patterns of the five newly produced mAbs show that immune complex immunization resulted in a generalized suppression of the immune response to most *Aspergillus* (Figure 1) and *Penicillium* species (Figure 2) when compared to mAb 14D6 which was produced against spores rather than immune complexes. While most of the ODs for mAb 14D6 were between 1 and 2.5, the ODs for all other mAbs were less than 0.25. However, immune complex immunization resulted in a marked increase in the cross-reactivities of the mAbs with other fungal species when compared to immunizing with spores only (Figure 3). Dematiaceous fungi (i.e. *Alternaria alternate*, *Epicoccum nigrum*, *Memnoniella echinata*, *Myrothecium verrucaria*, *Stachybotrys* spp. and *Ulocladium chartarum*) showed considerable cross-reactivity.

Figure 1. Monoclonal antibody reactivity with 16 *Aspergillus* and 1 *Eurotium* species. MAb 14D6 was produced against the particulate fraction of spores of *A. versicolor* and all other mAbs were produced against immune complexes composed of the particulate fraction of *A. versicolor* and antigen-reactive mAbs.

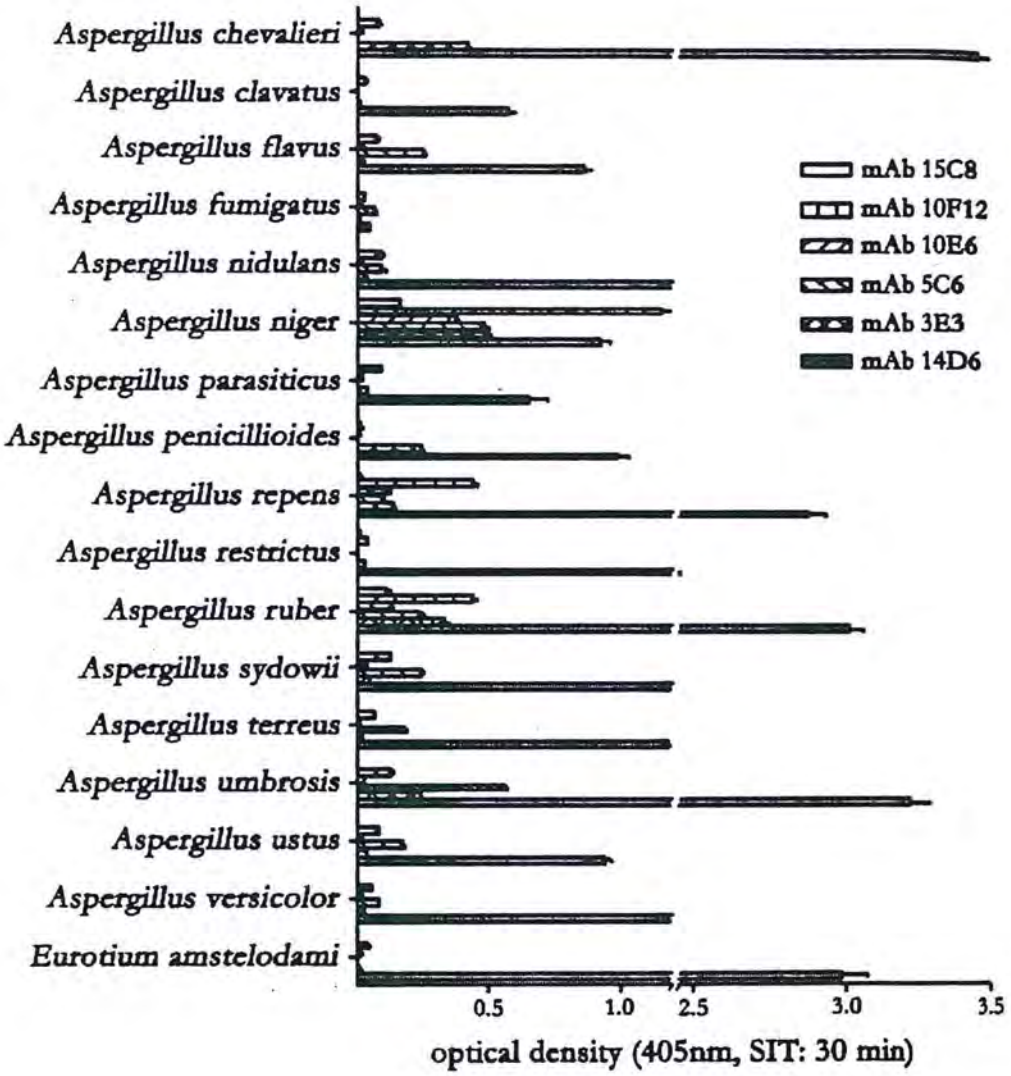


Figure 2. Monoclonal antibody reactivity with 14 *Penicillium* species. Mab 14D6 was produced against the particulate fraction of spores of *A. versicolor* and all other mAbs were produced against immune complexes composed of the particulate fraction of *A. versicolor* and antigen-reactive mAbs.

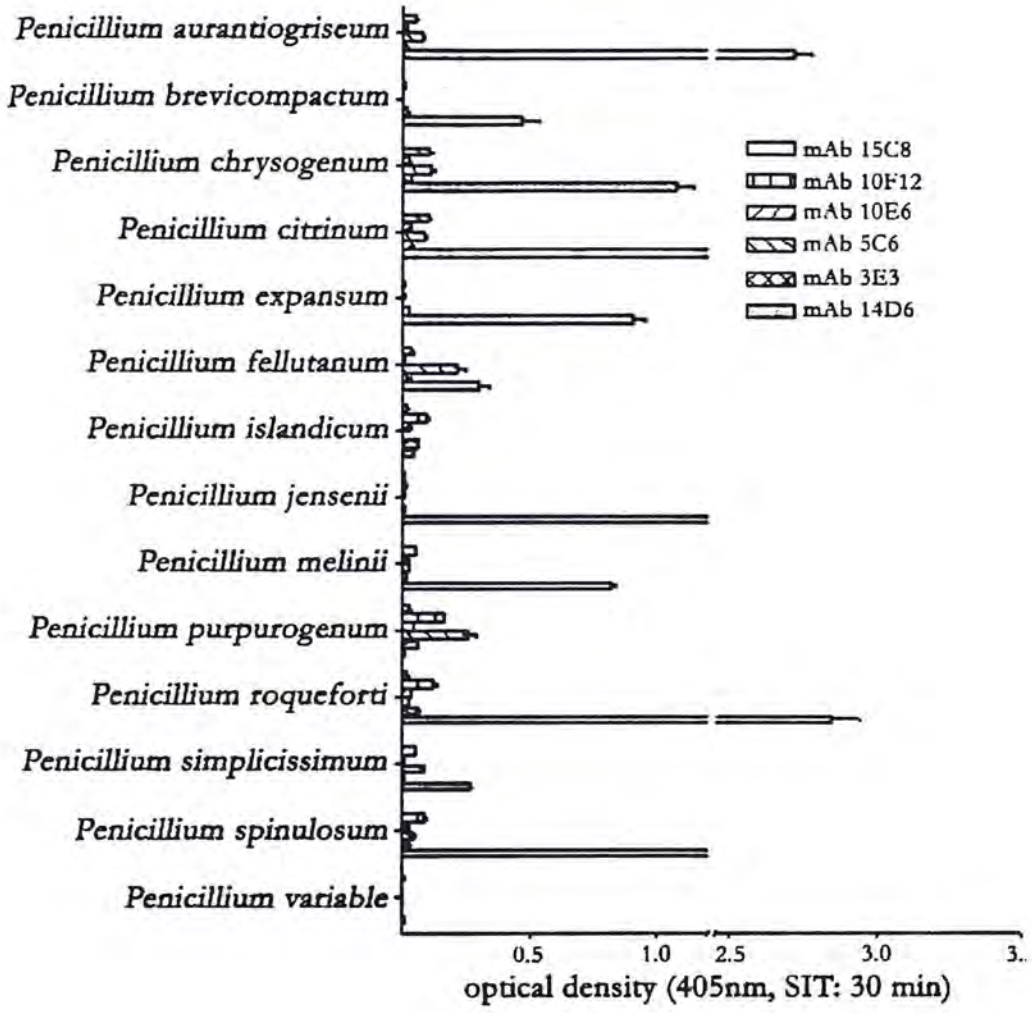
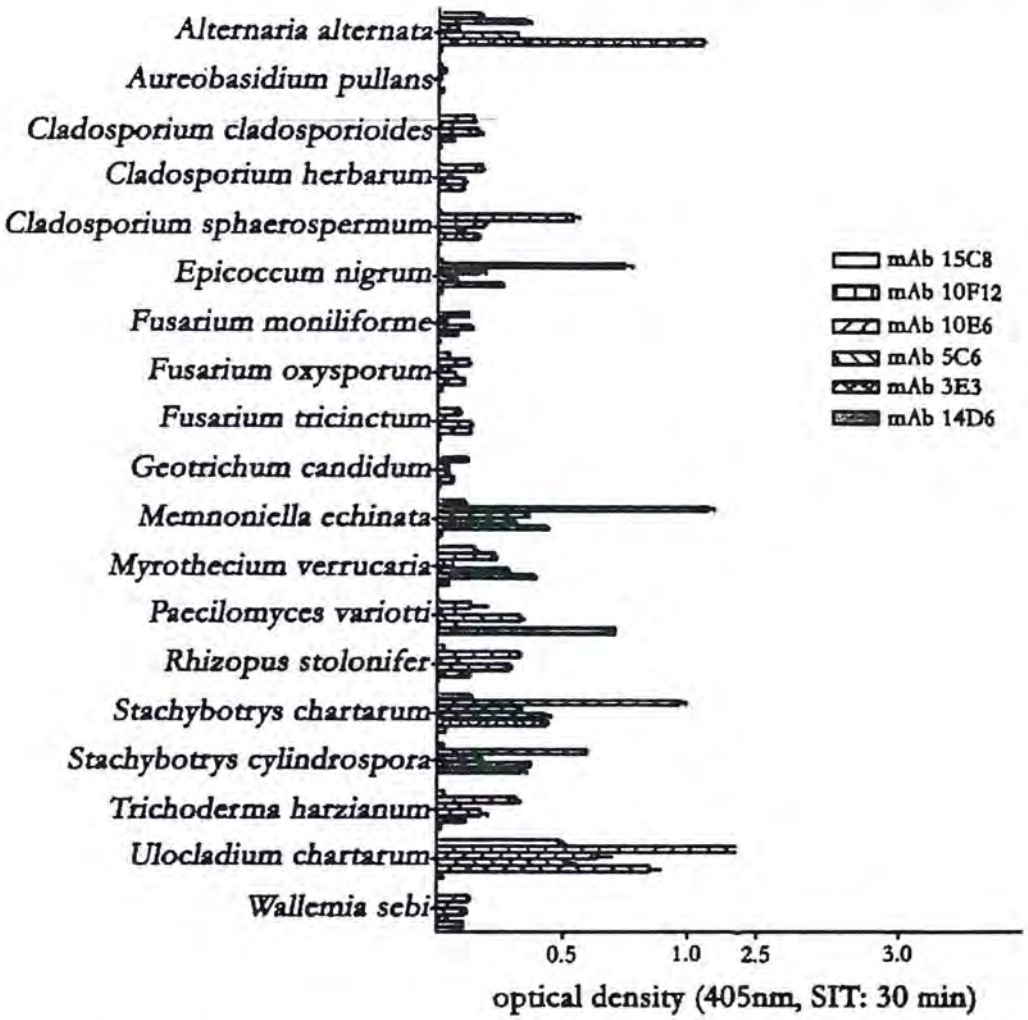


Figure 3. Monoclonal antibody reactivity with 19 fungal species commonly found in indoor environments. MAb 14D6 was produced against the particulate fraction of spores of *A. versicolor* and all other mAbs were produced against immune complexes composed of the particulate fraction of *A. versicolor* and antigen-reactive mAbs



DISCUSSION

The reduced cross-reactivity of all five mAbs with related *Aspergillus* and *Penicillium* species in combination with the failure to generate any species-specific mAbs suggests that the immunization of immune complexes results in a general state of immune suppression toward the target fungus as well as related fungi and does not increase the immunogenicity of target fungus-specific epitopes. Furthermore, the

increased cross-reactivity between many dematiaceous fungi and several *Aspergillus* and *Penicillium* species suggest that phylogenetically distant fungi share numerous common antibody binding sites and that the immunogenicity of the shared epitopes is more pronounced after the suppression of the genus-specific immune response. The highly variable reactivity of a given mAb with normalized amounts of spores of different fungi suggests that shared epitopes may be expressed at substantially different amounts on a per spore or unit biomass basis by different fungi. In related research, immune complex immunizations also failed to generate species-specific mAbs to *Stachybotrys chartarum* (Schmechel *et al.* 2003), *Penicillium brevicompactum* and *P. chrysogenum.*

CONCLUSIONS AND IMPLICATIONS

We conclude that immune complex immunization did not stimulate the production of species-specific mAbs. In order to generate such reagents, other tolerization methods such as neonatal tolerance or subtractive immunization using cyclophosphamide need to be investigated. Alternatively, other techniques such as synthetic or phage display of combinatorial antibody or peptide library techniques should be examined for their potential to provide desired detection reagents. We are currently using random peptide phage libraries to generate species-specific reagents to *A. versicolor* and other fungi.

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