

A monoclonal IgM directed against immunodominant catalase B of cell wall of *Aspergillus fumigatus* exerts anti-*A. fumigatus* activities

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Summary

Aspergillus fumigatus, a ubiquitous fungus, has been reported to cause human diseases like allergic pulmonary aspergillosis, aspergilloma and invasive infection. Limited spectrum and emergence of resistance has become a serious problem with available antifungals. Therefore, an alternative approach is required for successful treatment of mycoses. In the present study, immunogenic protein profile of *A. fumigatus* cell wall was generated using two-dimensional-gel electrophoresis and three hybridomas producing monoclonal antibodies (MAbs; IgM) were selected after fusion experiments. Of these three MAbs, MAb-7 exhibited potent *in vitro* inhibitory activity, which was confirmed by MTT assay, fluorescence-activated cell sorter analysis and immuno-fluorescence studies, and the protein was identified as catalase B using MALDI-TOF-MS.

Key words: *Aspergillus fumigatus*, monoclonal antibodies, catalase B, flow cytometry.

Introduction

Aspergillus fumigatus, a ubiquitous saprophytic fungus, is associated with over 80% of all human syndromes caused by aspergilli. Saprophytic presence to extensive invasion with this organism has been reported with various intermediary conditions depending on susceptibility and immunocompetence of the host¹ and the fungus is being identified as the most common aetiological agent among the species of *Aspergillus*-related diseases, which is of particular clinical importance. To combat the fungal infections, there are a number of antifungals available in the market. Unfortunately, none of these cover the whole spectrum of pathogenic *Aspergillus* species and are free of undesirable side-effects. Though there have been important

developments in antifungal agents on the basis of novel drug targets, new drugs are still required for the treatment of invasive aspergillosis because of the high incidence of therapeutic failure. The presently available antifungal drugs target mainly the inhibition of ergosterol synthesis.² However, the recent clinical launch of caspofungin, a β (1,3)-glucan synthase inhibitor, has confirmed that the development of new drugs can indeed be based on inhibition of cell-wall biosynthetic enzymes³ and a few such echinocandins and the glycolipid papulacandin inhibit the cell-wall synthesis, with which inhibition of cell-wall glucan biosynthesis leads to cessation of growth and cell lysis.⁴

The effect of immunoprotection in aspergillosis has experimentally been demonstrated through intravenous challenge (*A. fumigatus*) in mice by Lehman and White [5] in 1976. The identification and characterisation of cell-wall immunodominant proteins showing significant immune responses during aspergillosis could have essential ramification for developing novel diagnostic, prophylactic and therapeutics for aspergillosis. Recent studies have highlighted the therapeutic potential of monoclonal antibodies (MAbs) against *Aspergillus* infections,^{6,7} although the role of natural killer antibodies and total antibody response on antifungal immune

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resistance are not completely known, the use of antibodies is emerging as an effective adjunct therapy for fungal disease. Selective MAbs directed against specific epitopes are protective against local and systemic infections and may form the basis for immunotherapy. These antibodies can be used alone or in combination with other antifungal agents.⁸ In the present study, the immunodominant proteins of *A. fumigatus* cell wall were recognised on 2-D immunoblot using sera of patients suffering from aspergillosis. MAbs against cell-wall proteins of *A. fumigatus* were produced and characterised and the immunodominant proteins were identified using MALDI-TOF-MS.

Materials and methods

Fungal isolates and patient sera

Aspergillus fumigatus, a human isolate was used throughout the present study whereas the other pathogenic fungi used for cross-reactivity tests were *Aspergillus flavus*, *A. niger* DSM2182, *A. terreus* DSM826, *Candida albicans* ATCC10231, *C. krusei* ATCC6258, *C. parapsilosis* ATCC22019, *Cryptococcus neoformans* ATCC66031, *Sporothrix schenckii* and *Trichophyton mentagrophytes*. The species identity of these fungi was confirmed in our laboratory by amplifying the internal transcribed spacer regions of these fungi.⁹ Sera of 15 patients with aspergillosis (12 bronchopulmonary and three invasive) were collected from patients visiting local hospitals and confirmed by enzyme linked immunosorbent assay (ELISA; Sigma Chemical Co., St. Louis, MO, USA).

Isolation of cell-wall proteins

Aspergillus fumigatus mycelia harvested from 5-day-old shake cultures (in Sabouraud's dextrose broth at 300 rpm) were washed thoroughly with chilled, autoclaved triple-distilled water and vortexed in a Bead-Beater (Biospec Products Inc., Bartlesville, OK, USA) with glass beads in lysis buffer (100 mmol l⁻¹ Tris-HCl, pH 7.5) containing 1 mmol l⁻¹ EDTA, 5 mmol l⁻¹ dithiothreitol, 1 mmol l⁻¹ PMSF and 5 µg ml⁻¹ pepstatin A (all from Sigma Chemical Co.). After 15–20 cycles of 30 s each, complete breakage was monitored by phase-contrast microscopy. The broken mycelia were centrifuged at 5000 *g*, and the cell-wall pellet was boiled in extraction buffer containing 40 mmol l⁻¹ β-mercaptoethanol, 50 mmol l⁻¹ EDTA, 50 mmol l⁻¹ Tris-HCl and 2% (w/v) sodium dodecylsulphate (SDS). The resultant mixture was again centrifuged at 5000 *g* for 5 min and the supernatant was stored at –80 °C.

Two-dimensional (2-D) gel electrophoresis

Cell-wall pellet of *A. fumigatus* was suspended in an equal volume of readymade sample buffer (Sigma Chemical Co.), vortexed for 3–5 min at 4 °C, and tri-butyl-phosphine (TBP) was added to a final concentration of 5 mmol l⁻¹ and kept for 1 h. The generated thiols were alkylated for 2 h by addition of iodoacetamide (final concentration 15 mmol l⁻¹) and the protein concentration of the supernatant was determined using 2-D Quant Kit (Amersham Biosciences Corporation, San Francisco, CA, USA). Isoelectric focusing was carried out in carrier ampholytes (pH 4–10, Sigma Chemical Co.) in tube gels for a total of 10 000 Vh. For reproducibility, at least four tube gels having same diameter and length were run simultaneously. The gels were taken out in equilibrium buffer (125 mmol l⁻¹ Tris pH 6.8, 2% SDS, 10% glycerol and traces of bromophenol blue) and the second dimension isocratic separation was carried out by SDS–polyacrylamide gel electrophoresis following Laemmli's method of discontinuous buffer system.¹⁰ The images of gels were recorded on 'ImageScanner' (Amersham Biosciences) and the spots were matched by overlapping the images to confirm the reproducibility.

Monoclonal antibody production

Female BALB/c mice (6–8 weeks old) were immunised subcutaneously with 100 µl of cell-wall antigen in Freund's complete adjuvant (1 : 1), followed by five doses (days 7, 14, 21, 28 and 37) with Freund's incomplete adjuvant. A booster dose of the antigen in physiological saline was injected intravenously 3 days before fusion experiments. Prior clearance from the local animal ethics committee was obtained. Fusion of spleenocytes from immunised mouse with Sp₂/O myeloma cell line was performed in 50% polyethylene glycol in 96 well tissue culture plates as described elsewhere.⁷ The antibody producing hybridomas identified through ELISA were subcultured by limiting dilution to obtain single cell cultures and cryopreserved. Subsequently, the antibodies were produced in serum-free medium, precipitated with 50% ammonium sulphate, dialysed against Dulbecco's phosphate-buffered saline (DPBS) and stored at –80 °C.

Isotyping and cross-reactivity test

The isotype of the selected MAbs was determined with a monoclonal isotyping kit (Sigma Chemical Co.) according to the manufacturer's instructions. Cell wall from other

species of fungi namely *A. flavus*, *A. niger* DSM 2182, *A. terreus* DSM 826, *C. albicans* ATCC 10231, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, *C. neoformans* ATCC 66031, *S. schenckii* and *T. mentagrophytes* were isolated. The proteins from the cell wall were extracted by boiling in SDS-sample buffer and 20–40 µg of protein was loaded into the wells of the polyacrylamide gel. Electrophoresis and electro-transfer of proteins was performed as per standard protocol and the blot membrane was probed using all the three MAbs and developing blot with DAB reagent (Sigma Chemical Co.).

Immunoblot analysis

After separation on 2-D gels, the proteins were transferred onto polyvinylidene difluoride membrane in Towbin buffer (25 mmol l⁻¹ Tris, 192 mmol l⁻¹ glycine and 20% methanol)¹¹ at 50 mA. The transblotted membrane was incubated with blocking buffer [20 mmol l⁻¹ TBS, 0.1% (v/v) Tween-20, 5% (w/v) skimmed milk] for 18 h at 4 °C. After washing thrice with buffer (20 mmol l⁻¹ TBS, 0.1% Tween-20), the membranes were incubated at 37 °C for 2 h in pooled patient sera (1 : 100), immunoreactive spots were detected using HRP (horse radish Peroxidase)-labelled anti-human IgG, IgA, IgM and the blots were developed using diaminobenzidine.

In vitro inhibitory activity

Inhibitory activity of MAbs was determined by the method of Magliani *et al.* [12] with minor modifications. Briefly, 1 × 10³ spores of the test fungi were incubated with 50 µl of MAbs (1 mg ml⁻¹) for 18 h at 37 °C. An unspecific monoclonal IgM raised against *C. albicans* in our laboratory was used as a control. After incubation with MAbs, the colony forming unit (CFU) were determined by plating 50 µl of this suspension on Sabouraud's dextrose agar (SDA) plates at 35 °C.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

MTT assay for *in vitro* activity was performed by method described elsewhere.¹³ Briefly, 200 µl of suspension of swollen conidia (5 × 10⁴ cells ml⁻¹) in RPMI 1640 was incubated with 200 µl of MAbs (100 µg) in a 48-well tissue culture plate overnight at 37 °C in an incubator having an atmosphere of 5% CO₂. After centrifugation at 500 g the supernatant was discarded and 200 µl of MTT (0.05 mg ml⁻¹ in RPMI 1640) was added to each well and incubated for 3 h. The wells were dried and 200 µl of

DMSO was added and the absorbance of the dark formazan product was read at 550 nm. Wells with DMSO, swollen conidia or unspecific monoclonal IgM served as blank, positive and negative controls respectively.

Attachment inhibition with polypropylene

The cell adhesion assay was performed in 96-well ELISA plates. *Aspergillus fumigatus* conidia were harvested in DPBS with 0.05% tween 80 from 7 day old SDA slant cultures on and passed through sterilised glass wool to get a homogenous conidial suspension and after washing thrice with chilled DPBS suspended in the same (2 × 10³ cells ml⁻¹). To 100 µl of this suspension, 100 µl of antibody solution was added (100 µg ml⁻¹ final concentration) and dispensed in a microtitre plate. For control, DPBS was used in place of MAbs. The plate was incubated at 28 °C for 1 h and washed twice with DPBS. The number of conidia sticking to the plate was counted under an inverted phase-contrast microscope. The experiment was performed in triplicate and the data represent mean values.

FACS analysis

Spores of *A. fumigatus* were suspended in DPBS (1 × 10⁶ cells ml⁻¹). A total of 100 µg ml⁻¹ of MAbs in 1-ml aliquots of this cell suspension was incubated for 18 h at 37 °C. Unspecific antibody and DPBS alone were simultaneously used as controls. After incubation, 2 µl of propidium iodide (PI; 1 mg ml⁻¹ in water) and 10 µl of fluorescein diacetate (FDA; 5 mg ml⁻¹ in acetone diluted 1 : 20 in DPBS) were added, and the mixture was kept on ice for 30 min before analysing on a flow-cytometer (Becton Dickinson, San Jose, CA, USA). All MAbs at a concentration of 100 µg were pretreated with papain to cleave the Fc part by using the method of Rousseaux *et al.* [14] with slight modification; papain (Sigma Chemical Co.) was pre-activated with 10 mmol l⁻¹ cysteine in 1.25 mmol l⁻¹ EDTA for 20 min at 37 °C, and then mixed with MAb at 1 : 100 (w/w, enzyme : antibody) ratio and incubated at 37 °C for 12 h to determine the susceptibility of the cleavage. The reaction was stopped by the addition of iodoacetamide (25 mmol l⁻¹) and subjected to fluorescence-activated cell sorter (FACS) analysis with *A. fumigatus* spores as described above to subtract the amount of cell death because of agglutination, if any.¹⁴

Indirect immunofluorescence

Immunofluorescence experiments were performed using the protocol of Harris *et al.* [15] with slight

modification; 10 ml of complete medium 5% FCS (foetal calf serum) in RPMI 1640 was inoculated with 1×10^4 *A. fumigatus* conidia per ml in a petri plate containing poly-L-lysine coated glass cover slips and incubated at 37 °C. The cover slips with adherent fungal hyphae were fixed in 3.7% formaldehyde, 50 mmol l⁻¹ phosphate buffer (pH 7.0) and 0.2% Triton X-100 for 30–60 min, subsequently washed with water and thrice with PBS (pH 7.4), and 15 µl of test MAb (adjusted to 50 µg ml⁻¹ of PBS) was poured over them. The cover slips were incubated further in a moist chamber for 1–2 h, washed as above, incubated with FITC-conjugated goat anti-mouse IgM antibody (1 : 200) for 1–2 h, mounted in PBS containing 90% glycerol after washing and examined under a fluorescence microscope.

Identification of proteins by MALDI-TOF-MS

The immunoreactive protein spots (corresponding to reactivity of MAbs) were identified through overlapping the images of the blot and the gel. Subsequently, these spots were excised from 2-D gels and immersed in destaining solution (100 mmol l⁻¹ ammonium bicarbonate in 50% acetonitrile; ACN), and kept on a shaker for 30 min. The gel pieces were dehydrated with 200 µl of 100% ACN, dried on vacuum centrifuge for 30 min, washed alternately with 50 mmol l⁻¹ ammonium bicarbonate in 100% water and 50 mmol l⁻¹ ammonium bicarbonate in 50% ACN, and vacuum dried for 30 min. Sequencing grade trypsin (20 ng per vial) was dissolved in 100 µl of 10 mmol l⁻¹ HCl to which 900 µl of 100 mmol l⁻¹ ammonium bicarbonate prepared in 9% ACN was added. The dried gel pieces were kept on ice in 20 µl of the above mentioned trypsin solution and rehydrated for about 30 min. Then 20 µl of 100 mmol l⁻¹ ammonium bicarbonate was added to cover the gel pieces and incubated overnight at 37 °C after which 1% trifluoroacetic acid (TFA) was added. The microfuge tubes were then spun briefly and the supernatant was collected in a fresh, siliconised tubes. To the gel pieces [100 µl of 50% ACN, 0.2% TFA] was added and kept on a shaker for 30 min, and the supernatant was pooled. The volume of pooled supernatant was reduced to 2–3 µl on a vacuum evaporator and desalted using Zip-Tip columns (Millipore, Bedford, MA, USA) and the peptides were eluted with 0.1% formic acid.

One microlitre of above concentrated sample was mixed with an equal volume of matrix solution (10% α-cyano-4-hydroxycinnamic acid) and subjected to MALDI analysis. The peptide spectra were obtained on a Micromass TOF instrument (Tofspec. 2E Micromass,

Manchester, UK) in reflectron mode and the proteins were identified through MASCOT (<http://www.matrixscience.com>). These experiments were performed at least thrice to confirm our results.

Result

Immunoreactivity of *A. fumigatus* cell-wall proteins

The antigenic profile of *A. fumigatus* cell-wall proteins was analysed by 2-D gel electrophoresis (Fig. 1a) and Western blotting with pooled sera from patients as shown in Fig. 1b. A total of 36 antigenic determinants were spotted in case of pooled human sera with molecular masses from 35 to 95 kDa and pIs (isoelectric point) ranging from 4 to 10 of which 13 immunodominant proteins were identified by MALDI-TOF analysis (Table 2).

Monoclonal antibody production

A total of seven hybridomas were obtained after fusion experiments, which were further sub cloned and re-examined by ELISA. After three to four subcultures, three consistently MAb producing clones were identified through ELISA. These MAbs were designated as MAb-7, MAb-B and MAb-C. Supernatants of these three clones were collected and Western blot performed against cell-wall proteins separated by 2-D gel electrophoresis. Comparable binding to different protein spots was also detected with pooled patient sera (Fig. 1c).

Isotype determination and cross-reactivity of MAbs

The isotypes of MAbs were determined as IgM with MAb isotyping kit. All these MAbs (MAb-7, MAb-B and MAb-C) were found to be specific only for *A. fumigatus* as they did not cross react with cell-wall proteins of other fungi used in the present study.

Biological activity of MAbs

In vitro inhibitory activity

The MAb-7 exhibited a potent inhibitory activity against *A. fumigatus* at a concentration of 50 µg where it caused a significant reduction (76.6%) in the number of CFU compared with controls (with unspecific anti-Candida MAb and without any MAb) (Fig. 2a), while, MAb-C caused 39.0% reduction and MAb-B only 31.3%.

MTT assays and inhibition of attachment

The fungicidal activity of MAbs was also confirmed by MTT assay, where 21.18% ($P < 0.003$) hyphal damage

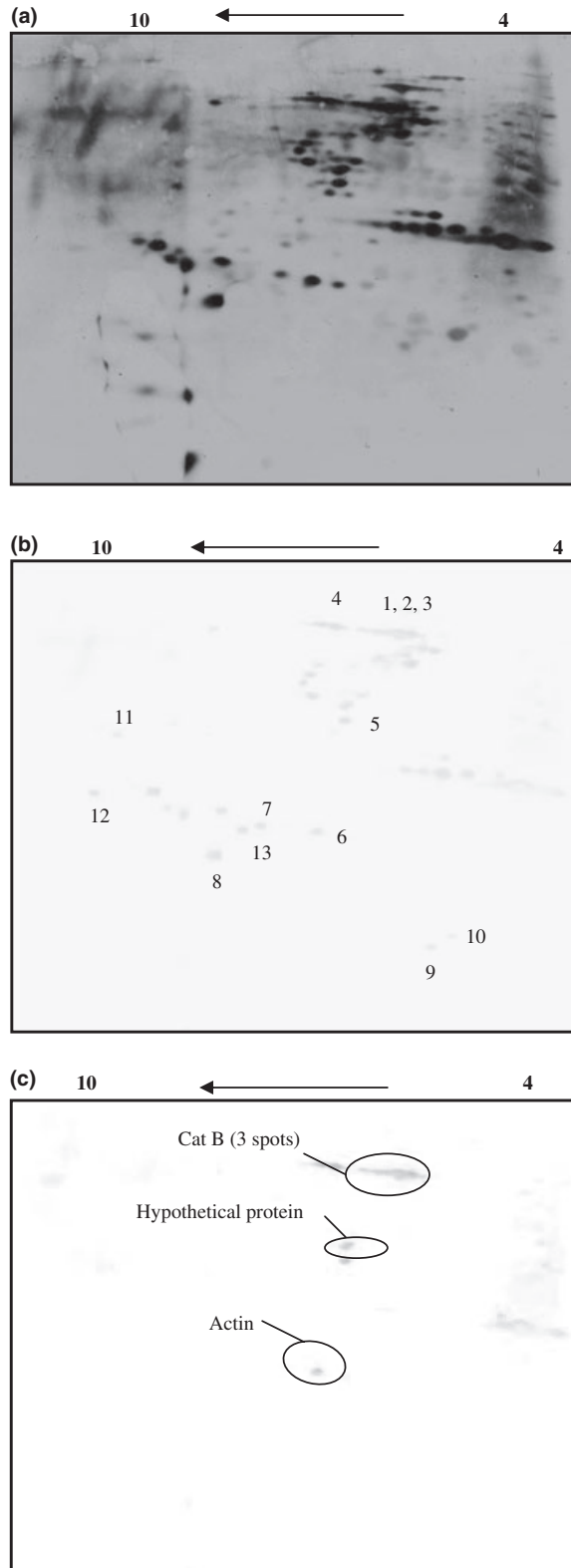


Figure 1 Antigenic profile of *Aspergillus fumigatus*. Cell wall proteins of *A. fumigatus* were extracted in readymade sample buffer (Sigma) and reduced with TBP followed by alkylation with iodoacetamide (IAA). A total of 100 µg of protein was loaded on 8-cm tube gel containing ampholytes pH 4–10 for isoelectric focussing. Second dimension run was performed in 12% isocratic gel and silver stained (a). Cell wall proteins were transferred on to the nitro-cellulose membrane and probed with pooled human *Aspergillus* positive sera and developed with DAB (b). Probed with all three monoclonal antibodies separately (c).

was observed with MAb-7, compared with the control (DPBS, 0%) and an unspecific MAb (8.1%). No significant hyphal damage was observed in case of MAb-C and MAb-B. Inhibition of attachment with polypropylene showed variable results for different MABs, where MAb-7 and MAb-C exhibited significant inhibition of attachment (Fig. 2b) to the wells as compared with DPBS and unspecific MAB while MAb-B did not exhibit any considerable inhibition of attachment.

FACS analysis

In order to confirm the cidal activity of MABs, the cells of *A. fumigatus* were incubated with MABs for 18 h and stained with PI and FDA. PI stained the dead cells while FDA stained both live and dead cells. The calculations for determining the per cent dead cells were made by using the formula, % dead cells = no. cells stained with PI (upper left quadrant) + no. cells stained with PI and FDA (upper right quadrant) × 100/total no. targeted cells. In 18 h, more than 70% of the cells were found to be dead that were treated with MAB-7 at a concentration of 100 µg and variable cidal activity was observed for other MABs (Fig. 2c). During the same period of time, amphotericin B (AMB) killed 93.5% cells at a concentration of 8 µg ml⁻¹ while unspecific antibody treatment resulted in 10.39% dead cells where comparatively larger percentage of unstained cells remained unexplained. The negative control DPBS did not show any significant effect (88% cells were alive).

Indirect immunofluorescence studies

Different localisation pattern was observed for all the three MABs (Fig. 3). MAb-7, MAb-B and MAb-C showed immunofluorescence on entire mycelial surface but in case of MAb-7, the intensity was greater at the apical region of *Aspergillus* mycelium (Fig. 3a–c). Immunofluorescence study showed that only MAb-7 had a capacity to get attached with mycelium and swollen conidia (Fig. 3a1), whereas in case of the other two MABs, binding was restricted to mycelium only.

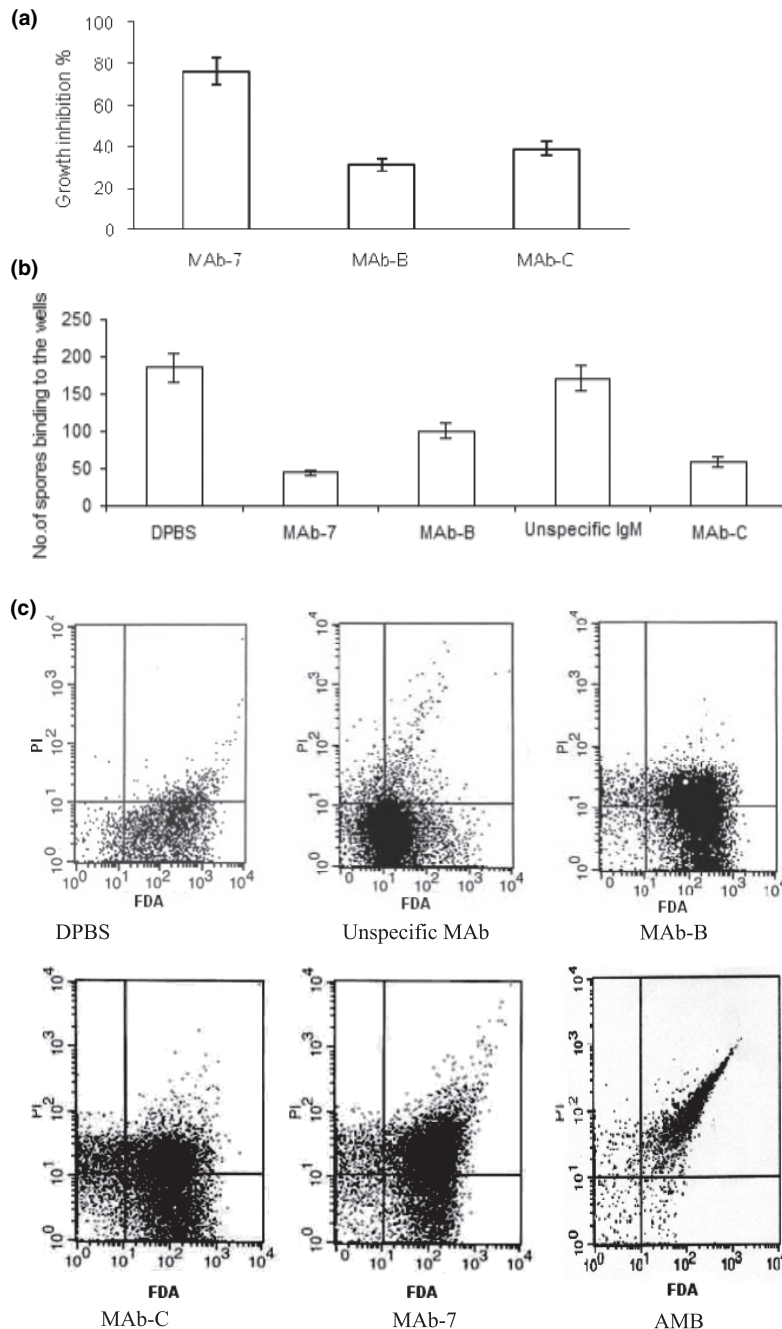


Figure 2 (a) *In vitro* activity. Fungicidal activity of monoclonal antibodies against *Aspergillus fumigatus* measured by the reduction in the number of CFU compared with the control with an unspecific MAb. Values are means of triplicate determinations $\pm$ standard errors of the means. Differences in fungicidal activity between *A. fumigatus* spores treated with MAb-7 and unspecific MAb were found to be statistically significant ($P < 0.001$) as determined by the Student's *t*-test. (b) Inhibition of attachment. Spores of *A. fumigatus* (2×10^3 cells ml^{-1}) were washed in DPBS and incubated with the tested monoclonal antibodies at the concentration of 50 μg each. 100 μl of this was added to the ELISA plate and kept in the incubator for 1 h at 37 °C. The wells were gently washed thrice with DPBS and cells binding to the wells were counted under the phase-contrast microscope. (c) FACS-analysis. Exponentially growing spores of *A. fumigatus* were exposed to all MABs at concentration of 100 μg (MAb-7, MAb-C, MAb-B), unspecific MAB, with DPBS only as a negative control and with AMB as a positive control for duration of 18 h at 37 °C, differentially stained for live and dead cells and analysed on flow cytometer.

Identification of proteins by MALDI-TOF

The peptide mass fingerprinting data obtained from MALDI-TOF (Fig. 4) were used to search for protein candidates in two sequence databases i.e. NCBI non-redundant protein database <http://www.ncbi.nlm.nih.gov> and a nearly complete *A. fumigatus* genome available at <http://www.cadre.man.ac.uk> using

MASCOT <http://www.matrixscience.com> and BIOEDIT software programs.

Two-dimensional gel electrophoresis showed several cell-wall proteins on the gel (Fig. 1a); MAb-7 exhibited binding at three spots all in a row whereas MAb-C and MAb-B showed binding at only one spot each (Fig. 1c). These binding proteins were identified as catalase B in case of MAb-7, a hypothetical protein and actin,

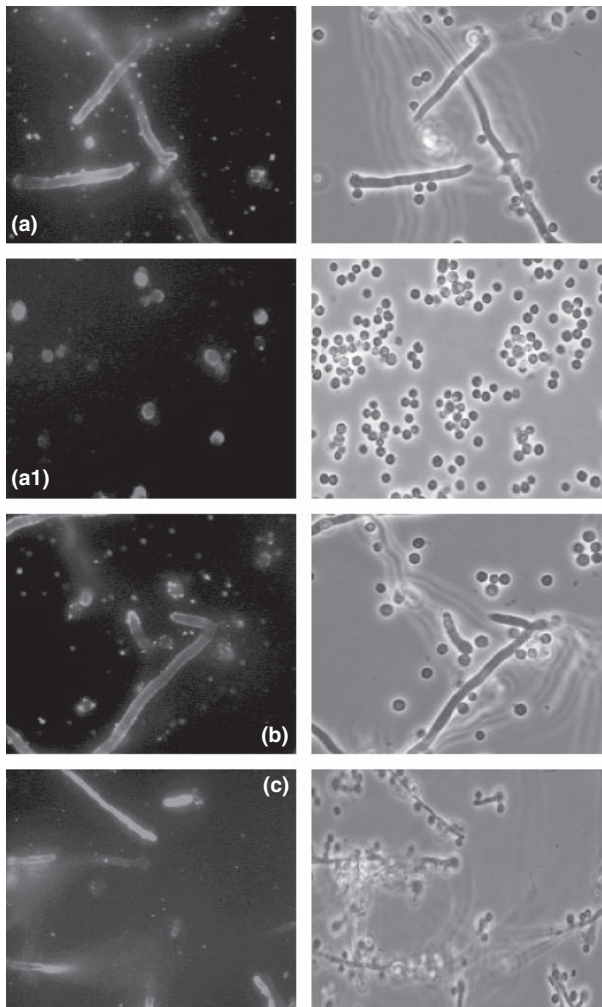


Figure 3 Immunofluorescence. MAbs show distinct patterns of immunolocalisation, *Aspergillus fumigatus* was grown for 14–16 h, fixed, incubated with primary MAb, followed by FITC-conjugated goat anti-mouse IgM secondary antibody. Primary antibodies: MAb-7, (a) bind with hyphae (a1) bind with swollen conidia: MAb-C (b) bind with hyphae only: and MAb-B (c) bind with hyphae only.

putative protein of *A. fumigatus* cell wall in case of MAb-C and MAb-B respectively (Fig. 4, Table 1). The other identified immunodominant proteins are listed in Table 2.

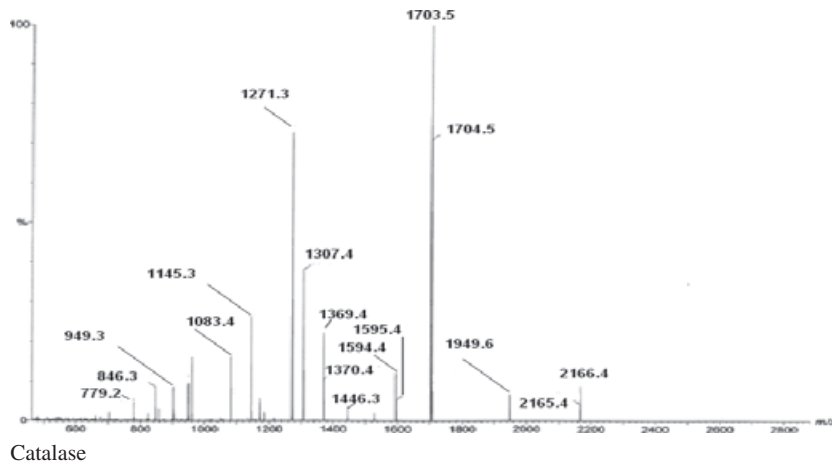
Discussion

The cell wall of *A. fumigatus* is a complex structure mainly composed of polysaccharides and where the majority of the antigens secreted by the fungus during its *in vitro* and *in vivo* growth are located.¹⁶ Some components of the cell wall are directly associated with

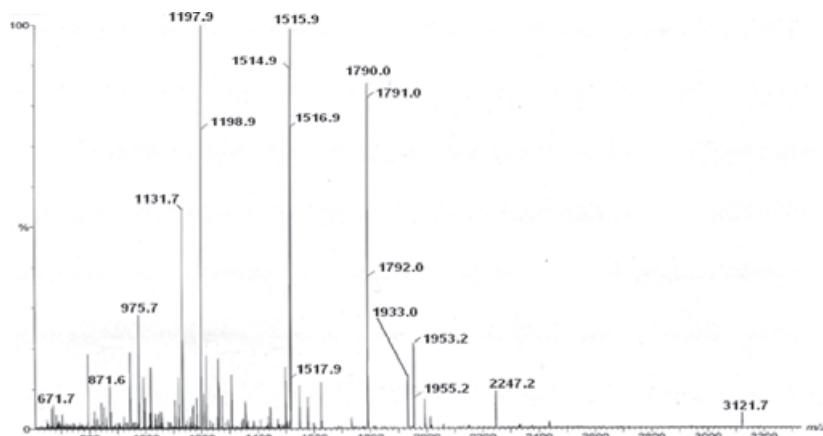
the colonisation of the host tissue and others with the damage to these tissues. *Aspergillus* produces catalase, an enzyme that breaks down H_2O_2 to water and oxygen. The strong association of aspergillosis with chronic granulomatous disease¹⁷ provides indirect evidence that catalase is a virulence factor. Cat1p/Cat B is one of the two catalase enzymes produced by the mycelium of *A. fumigatus* and is made up of four identical sub-units of 90-kDa glycosylated with mannose residue.

In this study, maximum cell-wall protein content was extracted with the use of a Bead-Beater compared with other described methods.¹⁸ Our results support that MAbs against specific target of cell wall may have protective value where the maximum *in vitro* activity was found in case of MAb-7 (Fig. 2a), formed against catalase B compared with MAb-B and MAb-C produced against actin putative protein and hypothetical protein of *A. fumigatus* cell wall that was confirmed by western blotting and MALDI-TOF. It is well known that this antigen is immunodominant (90 kDa).¹⁹ The 2-D blots probed with pooled patient sera resulted in identification of hyperimmune spots (three spots in a row) of catalase (Fig. 1c). The presence of catalase in conidia and mycelium both makes it more valuable for the immunotherapy as well as for immunodiagnosis.

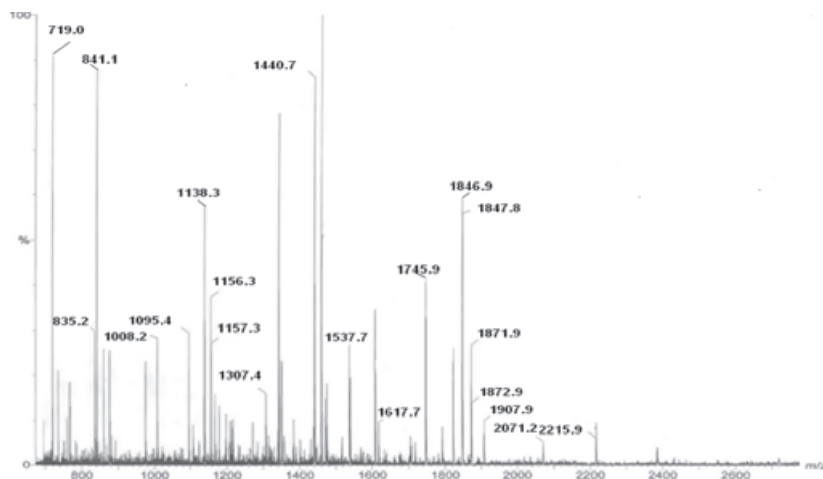
Cell-wall proteins are difficult to analyse because of their heterogeneity, interconnections with wall polysaccharides, low abundance, low solubility and hydrophobic nature. Bruneau *et al.* [20] adopted a proteomic approach to identify glycosylphosphatidylinositol-anchored proteins of *A. fumigatus*, which are involved in the organisation of the fungal cell wall. Recently, proteome analysis of major intracellular proteins of *A. fumigatus* by MALDI-MS was carried out,²¹ but immunogenic protein profile on 2-D gels of *A. fumigatus* cell wall was not described. Various putative virulence factors of *A. fumigatus* are presumed to contribute to its pathogenicity.²² The cell surface characteristics of resting conidia are altered during swelling and germination.²³ It is well established that the activities of any specific MAb rely on several variables, such as the characteristics of the targeted antigen, its role in the pathogenesis or in morphology. Because of continuous alteration of protein composition on the cell-surface binding of antibody to that particular cells gets affected. To check that the MAbs recognised cell-wall epitopes, they were used to label whole, fixed *A. fumigatus* germlings in indirect immunofluorescence experiments (Fig. 3). Our MAbs recognised true cell surface epitopes. Triton X-100 was used to rupture the cell wall to facilitate the entrance of all the three MAbs (pentameric



Catalase



Actin, putative



Hypothetical protein

Figure 4 Spectra. MALDI-TOF-MS spectra of *in situ* tryptic digested peptides obtained from respected protein spots, against which all three MAb's formed. Labelled peptides were used for database searches.

in nature) into the cell cytosol, yet none of the MAb's were able to bind with the cytosolic proteins (indirect immunofluorescence study) thereby indicating that

their epitopes were present on the cell surface only. All these MAb's showed distinct patterns of localisation, which revealed variation of these MAb's in binding to

Table 1 Characteristic features of all three monoclonal antibodies

MAbs	Isotype	Protein	Mol. wt. (kDa)	PI value	CADRE I.D.	Immunoreactivity	<i>In vitro</i> activity (%)	Immunofluorescence
MAB-7	IgM	Catalase B	79.9	5.60	Afu3g02270	++	76.6%	Entire surface, dense at the tip of mycelium, swollen conidia as well on mycelium
MAB-C	IgM	Hypothetical protein	67.6	5.73	Afu4g12350	+	39.0%	Surface only
MAB-B	IgM	Actin, putative	43.8	5.90	Afu6g04740	+	31.3%	Surface only

+, immunoreactive; ++, hyperimmunoreactive.

Table 2 Immunodominant proteins of *Aspergillus fumigatus* cell wall identified by MALDI-TOF- MS

S. no.	Spot no.	CADRE I.D.	NCBI I.D.	Mr kDa	pI	Protein name/proposed function
1	12	Afu1g10800	XP_752445	44.0	9.45	Thioesterase family protein
2	5	Afu1g13860	XP_752756	67.6	5.73	Conserved hypothetical protein
3	9	Afu2g10030	XP_755336	28.3	5.93	Vip1 protein
4	8	Afu2g10520	XP_755385	34.0	7.24	Urate oxydase
5	4	Afu2g13530	XP_755686	93.0	6.51	Translation elongation factor, EF-II subunit, putative similar to elongation factor II (<i>Pichia pastoris</i>)
6	13	Afu3g14710	XP_754154	41.0	6.60	Toxin biosynthesis protein putative, similar to <i>Fumonisin mycotoxin</i> biosynthesis protein
7	3	Afu3g02270	XP_748550	79.9	5.50	Catalase
8	2	Afu3g02270	XP_748550	79.9	5.60	Catalase
9	1	Afu3g02270	XP_748550	79.9	5.70	Catalase
10	11	Afu4g01370	XP_746321	61.0	9.10	Hypothetical protein
11	6	Afu6g04740	XP_747604	43.8	5.90	Actin, putative
12	7	Afu7g08450	XP_748770	49.3	6.24	Ornithine decarboxylase
13	10	Afu8g00550	XP_747150	29.5	5.21	Hypothetical protein

CADRE I.D., *A. fumigatus* gene annotation nomenclature according to Mabey *et al.* [24]. NCBI I.D., <http://www.ncbi.nlm.nih.gov>.

the epitope of *A. fumigatus* spores and mycelium. The maximum inhibitory activity shown by MAB-7 could be due to the binding activity of this antibody both with swollen conidia and mycelium. We selected intact germings for immunofluorescence study of mycelium. The restriction of MABs B and C to the hyphal tip could mean that the binding site or epitope is present only at the apical region or that the epitope is present in other regions, but accessible to the antibody only at the tip. To overcome this situation, we also observed immunofluorescence ability with swollen conidia; except MAB-7 (Fig. 3a1) rest of the two MABs did not bind with the conidia which means the epitope is common in two different morphological stages of *A. fumigatus*.

It is well known that antibodies help in agglutination of the cells that may result in false counting of cells on plates. To check whether the inhibitory activity exhibited by MAB-7 was not because of agglutinating property, another confirmatory assay, i.e. MTT assay was performed. This assay analysed the growth in terms

of ability of the live microorganisms to metabolise the MTT-dye where a maximum of 21.8% activity with MAB-7 was found. To confirm the inhibitory activity exhibited by MAB-7 and to observe the role of agglutination in cell death, FACS analysis was employed that is based on differential staining of live and dead cells followed by counting of these cells in a flow cytometer. Our FACS analysis confirmed that this inhibitory activity was not because of agglutination only, which was checked by using papain⁷ so that the Fc region required for agglutination is cleaved. All the three MABs showed activity (Fig. 2c), where only MAB-7 had the highest inhibitory activity. In FACS analysis, AMB was included as the control where 93.5% killing of *A. fumigatus* was observed which also confirmed the suitability of FACS analysis in determining the cidal effect.

In conclusion, MAB-7 may be useful in immunodiagnosis and also in immunotherapy as our results indicated *in vitro*. Proteome analysis of *A. fumigatus*

cell-wall proteins may play an important role in the identification of the specific proteins, which have important role in the initial morphology and development. Therefore, cell-wall antigens (immunodominant) constitute candidates for immunotherapeutic development. Hence, MAb production against such useful proteins could be the foundation for immunotherapy and one of the best-suited alternatives to overcome the antifungal resistance.

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