Candidacidal activity of a monoclonal antibody that binds with glycosyl moieties of proteins of *Candida albicans*

AMOL KAVISHWAR & P. K. SHUKLA
Fermentation Technology Division, Central Drug Research Institute, Lucknow, India

The incidence of life threatening mycoses caused by opportunistic fungi has increased dramatically in recent years with *Candida* and *Aspergillus* being the most commonly encountered species. *Candida albicans* ranks among the four most common causes of bloodstream infections and is responsible for vulvovaginal candidiasis in the majority of women in their reproductive years. Limited spectrum of antifungal activity of currently available antifungals and emergence of resistance has become a serious problem. Therefore, in search of an alternative form of treatment of candidiasis, in the present study a monoclonal antibody (MAb-G5) of IgA isotype was identified from the hybridoma produced by the fusion of lymphocytes of *C. albicans* immunized mouse with Sp2/O cells. The MAb-G5 exhibited *in vitro* candidacidal activity and was also found to be useful for treatment and prophylactic use under experimental conditions *in vivo*.

**Keywords** *Candida albicans*, Monoclonal IgA, candidacidal, vaginal candidiasis, flow cytometry

**Introduction**

*Candida albicans*, is an opportunistic pathogen that normally colonizes mucosal surfaces of humans. Depending on the underlying host defects *C. albicans* is able to cause a variety of infections that range from mucosal to life threatening disseminated candidiasis [1]. Mucosal candidiasis is a common complication of HIV infection; where more than 90% individuals with progressive HIV disease develop oropharyngeal candidiasis at some stage during the course of their disease [2]. About 75% of otherwise healthy females encounter at least one episode of vaginal candidiasis during their reproductive life and about 5% of them suffer from recurrent vulvo-vaginal candidiasis [3]. Currently available antifungals act on targets also found in mammalian cells, which may result in toxicity or an adverse drug interaction. Over and above emergence of resistant *C. albicans* strains in patients, receiving triazole treatment has become a serious problem. Combination of various antifungal agents with each other and with other molecules such as peptides has shown encouraging results [4] but *in vivo* studies are not encouraging. Therefore, in search of an alternative form of treatment of candidiasis, the last decade has seen an upsurge in the interest in candidacidal antibody directed against specific epitopes of *C. albicans*.

Although the role of humoral immunity in host defense against fungi has been controversial, monoclonal/polyclonal antibodies directed against specific epitope of fungi were shown to be protective in animal models of infection. Monoclonal antibodies that mimic the image of yeast killer toxin [5] and bind with the β-glucan of killer toxin receptor [6], and another monoclonal antibody-C7 [7] raised against the main target (>200 kDa) of salivary sIgA in the cell wall of *C. albicans*, are reported to be candidacidal. Some proteins like mannose binding protein (MBP) and protein D that bind with the carbohydrate moiety are also candidacidal [8,9]. Besides directly cidal antibodies, monoclonal antibodies directed against secreted aspartyl proteinase (Sap) and mannosyl part of cell surface mannoprotein offer protection in animal models of candidiasis [10]. However, not all antibodies...
that are directed against mannosyl moiety were found to be protective. Fine structure of mannose moiety is very complex and carries many epitopes against which antibodies are produced [11]. Monoclonal antibodies (B6.1, and G3) [12,13] that bind with the acid-labile moiety were protective while another antibody (B6) that binds with acid-stable fraction failed to protect mice against disseminated candidiasis [14]. Recently a humanized monoclonal antibody (Mycograb®) directed against Hsp90 [15] has entered clinical trials for the treatment of candidiasis. Thus, it seems that the presence of high titer of agglutinins in serum may not protect an individual against candidiasis [16] but antibodies directed against specific epitopes may be developed for use in antifungal therapy.

In the present study, we developed monoclonal antibodies against cell wall proteins of C. albicans and identified one candidacidal monoclonal antibody (MAb-G5) that was of IgA isotype. This antibody was found effective for the treatment of vaginal candidiasis by local route in experimental mouse model. Interestingly the antibody when administered intravenously was transported to the vaginal lumen and protected experimental mouse against vaginal candidiasis.

Materials and methods

Organism and culture conditions

C. albicans (ATCC 10231) was maintained on Sabouraud dextrose agar (SDA, Difco Laboratories) slants at 4°C. Cells were grown for 16 h in Sabouraud dextrose broth at 28°C, with orbital shaking at 200 rpm before use. Prior to each experiment, cells were collected by centrifugation, washed in chilled Dulbecco’s PBS (DPBS; Sigma) and suspended in the same and stored on ice for not more than 15 min before use. Other strains used in cross-reactivity studies were C. parapsilosis (ATCC 22019), Cryptococcus neoformans (ATCC 66031), Fusarium solani and Trichophyton mentagrophytes. The species identity of these fungi was further confirmed in our lab by amplifying ITS region of these fungi [17].

Mice

Female BALB/c mice (8–9 weeks) were used in the present study. All animals were housed in the animal care facility of the institute according to the norms laid down by animal ethics committee of the institute. Pseudo-estrous condition in animals was induced by injecting 500 µg of oestradiol benzoate, subcutaneously, three days before the infection was given [18].

Crude cell wall preparation

Exponentially growing cells of C. albicans (ATCC 10231) were harvested by centrifugation and washed thrice in chilled distilled water. The pellet was resuspended in chilled 20 mM Tris-C1 (pH 7.4), supplemented with protease inhibitor cocktail (PMSF 1 mM, EDTA 1 mM, AEBSF (4-[(2-Aminoethyl)benzenesulphonyl fluoride] 1 mM, 1,10-Phenanthroline 5 mM, Pepstatin A 20 µM, E-64 10 µM), to a cell density of 10^6 cells/ml. The cell suspension was then mixed with equal volume of glass beads and vortexed vigorously in Bead-Beater (Biospec Products Inc, USA). After 10–12 cycles of 1 min each with a pause of 3 min, cell breakage was observed under phase-contrast microscope. The lysate was also plated onto SDA plates to confirm the absence of any live cells. Cell wall was collected by centrifugation, washed in chilled distilled water, and stored at −80°C until further use. For cross reactivity studies cell wall of C. parapsilosis, Cryptococcus neoformans, F. solani and T. mentagrophytes was isolated and stored as above.

Production of monoclonal antibodies

Crude cell wall of C. albicans was resuspended in DPBS and protein value was determined according to standard methods. The protein value of cell wall suspension was adjusted to 2 mg/ml and mixed with equal volume of Freund’s complete adjuvant (Sigma). Female BALB/c mice were immunized intraperitonially with 50 µl of this preparation. Equal amount of two boosters were also given separated by an interval of 15 days in Freund’s incomplete adjuvant. One month after the last booster, the mice were injected intravenously with 50 µl of cell wall suspension and sacrificed on day 7. The spleen was removed and fusion with Sp2/O was performed according to standard protocols [19]. Ten days after fusion, the supernatants from 96-well cell culture plates with growing hybridoma were screened for production of monoclonal antibodies by ELISA as described below. The positive hybrids were immediately sub-cloned, twice, by serial dilution and reconfirmed by ELISA. All clones were preserved at −80°C.

ELISA

Crude cell wall suspension of C. albicans (100 µl) was mixed with 300 µl of 0.06 M Tris-C1 pH 6.8, containing 2% SDS and 5% BME (β-mercaptoethanol) and incubated at 95°C for 4 min. The suspension was cooled to RT, centrifuged at 10,000 g for 5 min and the protein value of supernatant was determined using 2-D Quant Kit (Amersham, USA). The supernatant was
diluted in 0.6 M bicarbonate buffer, pH 9.6 to adjust the concentration of protein to 20 μg/ml. Micro-titer plates (Greiner Bio One GmbH) were coated with 50 μl/well of prepared antigen and incubated at 4°C for 16 h. Plates were washed twice with PBST (50 mM phosphate buffered saline, pH 7.2 containing 0.05% Tween 20) and blocked with 100 μl of 1% BSA (fraction V, Sigma) in PBST for 90 min at RT. After washing the plates 50 μl of test hybridoma cell culture supernatant was added to each well and incubated at 37°C for 90 min. Secondary antibody (1:10,000 of each, peroxidase-conjugated anti-mouse IgA Cat. No. A-4789, anti-mouse IgG Cat. No. A-9044 and anti-mouse IgM Cat. No. A-8786, Sigma) was added to each well (50 μl) after washing the plates and incubated for 90 min. Then 50 μl of substrate solution (O-phenylenediamine dihydrochloride in 0.05 M phosphate-citrate buffer, pH 5.0) was added to each well of thoroughly washed plates and incubated at 37°C for 30 min. The reaction was stopped with 50 μl of 7% sulfuric acid and the optical density (OD) was measured at 450 nm on microplate reader (Molecular Devices, USA). For controls, Sp2/O culture supernatant was used as primary antibody. An OD of 0.5 was considered as positive (after subtracting the OD of Sp2/O culture supernatant).

Free-phase inhibition ELISA

Proteins of C. albicans were separated by SDS-PAGE (8 x 10 cm gel) and transferred onto PVDF membrane. Two such membranes were washed with two changes of TDW and incubated in 10 ml solution of 50 mM sodium periodate (in 50 mM acetate buffer, pH 4.5) for 16 h at 4°C with mild agitation. The resultant solution was lyophilized to 1 ml and passed through Sephadex-G25 column. The eluate was lyophilized, and reconstituted in 200 μl of PBS. The carbohydrate content of the reconstituted eluate was determined by the method of Dubois et al. [20]. Fifty micro liter of this was serially diluted (two-fold) in PBST in an ELISA plate coated with C. albicans proteins as described above. To these wells, 50 μl of MAb-G5 (1:5000 in PBST) was added and incubated with mild agitation at 37°C. After incubation the wells were washed thrice in PBS and developed with OPD. Wells with no isolated carbohydrate was taken as positive control, and well with no primary antibody was taken as negative controls. Other controls included wells with no secondary antibody, and wells with neither carbohydrate nor primary antibody. After reading the plate at 490 nm, the OD of the well with neither carbohydrate nor primary antibody was subtracted from all the values and an OD of 0.3 was considered significant. The data represents average of one experiment performed in triplicate.

Isotype confirmation of Mab

The isotype of MAb-G5 was determined by antibody capture assay using the ImmunoType kit (Sigma) as per instructions of the manufacturer.

Purification of monoclonal antibody (MAb)

For all experiments MAb-G5 was produced in serum-free and protein free hybridoma medium (S-2772, Sigma). Culture supernatant was collected by centrifugation at 1,000 g for 5 min and then further cleared at 18,000 g for 5 min. Antibodies from the medium were concentrated by 50% ammonium sulfate precipitation, followed by extensive dialysis against DPBS using Mini Dialysis Kit (8 kDa cut-off, Amersham). For controls, fusion partner Sp2/O and an irrelevant monoclonal IgA (irrelevant-MAb) produced by a hybridoma line from the same fusion experiment and not binding with cell wall proteins of C. albicans in ELISA or western blot was also grown in serum-free medium and the supernatant was similarly concentrated. This irrelevant MAB did not recognize intact Candida cells or other fungal elements as determined by immunofluorescence assay as given below. Protein values were determined by the method of Lowry et al. [21].

Epitope localization on the surface of Candida albicans

Exponentially growing cells of C. albicans were harvested by centrifugation, washed thrice in chilled distilled water and resuspended to 1 x 10⁶ cells/ml in RPMI 1640 supplemented with 10% FBS and incubated at 37°C. After 1–2 h, cell suspension was observed under phase contrast microscope and cells were fixed by addition of formaldehyde to a final concentration of 4% and stored at 4°C. After 30 min, cells were pelleted and washed with chilled DPBS and suspended in the same to a cell density of 1 x 10⁵ cells/ml. To 100 μl of this cell suspension an equal amount of antibody (10 μg) was added and incubated at 4°C. After 1 h the cells were pelleted and washed thrice in chilled DPBS and 100 μl of 1: 100 dilution of FITC-conjugated anti-mouse antibody was added to it. The cell suspension was incubated at 4°C for 1 h and washed with chilled DPBS. To the pellet 100 μl of 90% glycerol buffered with DPBS containing traces of
p-phenylenediamine (PPD) was added and kept overnight at 4°C. The epifluorescence was observed under a phase-contrast fluorescence microscope.

**Gel electrophoresis and western blotting**

Cell wall pellet (100 μl) was suspended in 300 μl of SDS sample buffer [22] (2% SDS, 5% BME, 10% glycerol, and traces of bromophenol blue in 0.06 M Tris-Cl, pH 6.8) and heated at 95°C for 4 min in a water bath. A 15–30 μg of protein was loaded per well on a 12% discontinuous acrylamide gel [23] and electrophoresed at a constant current of 10 mA. After electrophoresis, proteins in gels were stained either with Coomassie blue or periodic acid Schiff (PAS) [24] or electro-transferred onto nitrocellulose or PVDF membranes followed by staining with Ponceau S.

After washing off Ponceau S, the membranes were blocked with 1% low-fat skimmed milk prepared in TBST (0.8% NaCl and 0.01% Tween 20 in 20 mM Tris-Cl, pH 7.5) and immunoblot was performed by incubating membrane with 10 μg of test antibody in 10 ml of TBST. Controls included no primary antibody, and Sp2/O culture supernatant. Peroxidase-conjugated antimouse IgA, IgG and IgM (diluted 1: 10,000 in RPMI) was added and incubated for 1 h. This was centrifuged and 100 μl of DMSO was added to the pellet and the absorbance of the dark formazan product was read at 550 nm. All controls and concentrations were kept the same as in the above experiment. The optical density obtained for amphotericin B was considered as 100% inhibition, and that of DPBS was 0%. The formula used to calculate percent inhibition was \( \frac{100 - (x - OD_{AMB})/OD_{DPBS} - OD_{AMB} \times 100} {\ldots} \), where x is the OD of test antibody.

**Cell viability assay**

Exponentially growing cells of *C. albicans* were collected and after washing suspended in DPBS at a cell density of 1 × 10⁵ cells/ml. To 500 μl of this cell suspension, an equal amount of test antibody was added and incubated for 1 h at 28°C. Similar controls as described above were used in this experiment. After incubation, the cells were centrifuged at 1 × 1,000 g for 5 min and to the pellet 100 μl of MTT (0.5 mg/ml in RPMI) was added and incubated for 1 h. This was centrifuged and 100 μl of DMSO was added to the pellet and the absorbance of the dark formazan product was read at 550 nm. All controls and concentrations were kept the same as in the above experiment. The optical density obtained for amphotericin B was considered as 100% inhibition, and that of DPBS was 0%. The formula used to calculate percent inhibition was \( \frac{100 - (x - OD_{AMB})/OD_{DPBS} - OD_{AMB} \times 100} {\ldots} \), where x is the OD of test antibody.

**Candidacidal activity of MAb-G5**

Cell suspension of *C. albicans* in DPBS was adjusted approximately to 1 × 10⁶ cells/ml and 100 μl of this was serially diluted and plated on SCA (SDA containing 50 mg/l chloramphenicol) plates to determine exact number of viable cells. One hundred μl from the same cell suspension was incubated with 100 μl of test antibody solution (100 μg/ml) for 16 h [25] at 35°C, and diluted serially and spread on SCA plates. Controls included DPBS, Sp2/O cell culture supernatant (100 μg/ml), an irrelevant IgA (100 μg/ml), and amphotericin B (18 μg/ml). Values quoted represent mean of assays performed in duplicate on at least two different occasions.

**MTT assay**

Candidacidal activity of MAb-G5 was studied by MTT reduction assay. Briefly, 100 μl of cell suspension (~1 × 10⁴ cells/ml) was incubated with 100 μl of antibody (10 μg) and incubated for 16 h at 35°C. After incubation, cells were centrifuged at 1 × 1,000 g for 5 min and to the pellet 100 μl of MTT (0.5 mg/ml in RPMI) was added and incubated for 1 h. This was centrifuged and 100 μl of DMSO was added to the pellet and the absorbance of the dark formazan product was read at 550 nm. All controls and concentrations were kept the same as in the above experiment. The optical density obtained for amphotericin B was considered as 100% inhibition, and that of DPBS was 0%. The formula used to calculate percent inhibition was \( \frac{100 - (x - OD_{AMB})/OD_{DPBS} - OD_{AMB} \times 100} {\ldots} \), where x is the OD of test antibody.

**Protection against C. albicans vaginal challenge**

At least five mice were used in each group. Female BALB/c mice in pseudo-estrous cycle were challenged intravaginally with 3 × 10⁵ cells in 20 μl of DPBS. After every 24 h the mice were treated intravaginally with 20 μl of antibody (1 mg/ml) in DPBS for three days starting 24 h after the infection was given. Vaginal lavages were collected on days 0, 1, 2, 3, and 5 by washing vaginal lumen with 20 μl of DPBS before administration of MAb-G5. The viable cells in the collected lavages were counted by two-fold serial
dilution and plating onto SCA plates, and expressed as CFU/ml of vaginal lavages. Controls included mice treated with DPBS, and an irrelevant monoclonal IgA (1 mg/ml). In one group of five mice the CFU was evaluated 30 min after they were challenged to estimate the number of cells that were initially present. This group was not used in any subsequent experiments.

In another set of experiment, mice in pseudo-estrous cycle received intravenously, a single dose of 100 μl of antibody (1 mg/ml) prophylactically 2 h before they were challenged intravaginally with C. albicans. Collection of vaginal lavages was done on days 1, 2, 3, and 5 essentially in the same manner as described above. Control treatments were the same as described above except that administration (100 μl) was once through the intravenous route.

Differences between the control and treated population were ascertained by performing the test of equality (Student’s t-test) of two population means and also by ANOVA on Ranks.

In yet another experiment, two mice received 100 μl of DPBS, intravenously and vaginal lavages were collected as above after every 30 min for 5 h and pooled. On the next day, both mice received 100 μl of MAb-G5 intravenously and vaginal lavages were collected and pooled. Protein value was estimated and adjusted to 1 mg/ml with DPBS. An ELISA was performed using these vaginal lavages as primary antibody against cell wall proteins of C. albicans essentially as described above.

**Results**

**Isolation of MAb**

Fusion of lymphocytes from the spleen of immunized mouse with Sp2/O myeloma cells resulted in a number of hybridoma cell lines. Subsequent sub-cloning of ELISA positive hybridomas followed by western blot using cell wall proteins of C. albicans led to the identification of MAb-G5 that was found to bind with many proteins (Fig. 1) and was selected for further studies. The isotype of this MAb-G5 was found to be IgA (Fig. 2). The antibody was found to completely opsonize conidia of C. albicans but the presence of this epitope on mycelial surface was rather patchy. The irrelevant-MAb used, as control did not bind to any cell surface protein (Fig. 3).

**Epitope specificity of MAb-G5**

It is known that many proteins of cell wall of C. albicans are post-translationally modified. Thus, cell wall proteins of C. albicans transblotted onto nitrocellulose membrane were subjected to de-glycosylation and probed with MAb-G5. No protein was recognized after sodium periodate treatment of blotted proteins on nitrocellulose membrane by immunoblot while another such membrane without sodium periodate treatment and probed in the same manner exhibited to retain the binding capacity. This was further confirmed by the loss of binding capacity of peroxidase-conjugated concanavalin A with deglycosylated proteins of C. albicans; indicating that de-glycosylation of proteins was complete after 20 h incubation. All bands that were recognized by MAb-G5 did not react with ConA peroxidase. In order to verify the presence of carbohydrate moiety in these bands, PAS staining was performed. The bands that were recognized by MAb-G5 were stained pink by PAS staining, indicating towards the presence of carbohydrate moiety. Another antibody NE-5 used as control exhibited binding with C. albicans protein even after deglycosylation indicating that sodium periodate treatment does not degrade proteins. This was further confirmed by Coomassie staining of the oxidized proteins on blots that did not exhibit any variation in the banding pattern. MAb-G5

![Fig. 1](image1.png) Proteins from crude cell wall of *Candida albicans* were separated by SDS-PAGE and stained with coomassie (1) and PAS (2), and probed with MAb-G5 (3), peroxidase-conjugated concanavalin A (4), and MAb-NE5 (5). Proteins on membrane (NCP/PVDF) were subjected to de-glycosylation and probed with MAb-G5 (6), concanavalin A (7) and MAb-NE5 (8) or stained with Commassie (9) indicating intact proteins. Epitope of MAb-G5 and concanavalin A was lost due to de-glycosylation but the activity of NE5 was retained.

![Fig. 2](image2.png) ImmunoTyping kit (Sigma) was used to confirm the isotype of MAb-G5.
was found to cross react with *C. parapsilosis*, but not with other fungi tested.

**Free-phase inhibition ELISA**

Deglycosylation of cell wall proteins of *C. albicans* with sodium periodate was performed and the released carbohydrates were reconstituted to 169 µg/ml of glucose equivalent in PBS. A dose-dependent inhibition of MAb-G5 was observed in free phase inhibition ELISA.

**Candidacidal activity of MAb**

MAb-G5 was found to exhibit direct *Candida* candidacidal activity. Incubation of *C. albicans* cells (1 × 10⁵ cells) with MAb-G5 (10 µg), for 16 h at 35°C, resulted in 79% reduction of CFU as compared to DPBS. No significant candidal activity was observed in Sp2/O culture supernatant or an irrelevant antibody of the IgA isotype, purified similarly and used at same concentrations as MAb-G5. Amphotericin B killed all cells in the given period (Fig. 4). The figure shows average values with standard deviation.

Fungicidal activity of MAb-G5 was also confirmed by MTT reduction assay where a reduction of 86.3% in viability was observed when compared with DPBS (Fig. 5). A minimal inhibition of 11% and 28.4% was also observed for both the controls, Sp2/O and irrelevant MAb respectively. The figure represents average data from one of the three experiments performed in triplicate.

To confirm the candidacidal activity of MAb-G5, the cells of *C. albicans* were incubated in the presence of antibodies for 1 h, and stained with propidium iodide and fluorescein diacetate. PI stained the dead cells while FDA stained both live and dead cells. All the positive and negative controls taken were same as above. In 1 h, more than 63% of the cells were found to be stained with PI and less than 4% stained with FDA, indicating that MAb-G5 killed all the cells.

*Fig. 3* Immunofluorescent (a and c) and bright field photomicrographs (b and d) of the same microscopic fields of *Candida albicans* grown in RPMI 1640 supplemented with 10% FBS for 1–2 h and stained with MAb-G5 (a and b) or irrelevant-MAb (c and d). Note the patchy distribution of fluorescence on filament.

*Fig. 4* In-vitro candidacidal activity of MAb-G5. Approximately 1 × 10⁵ cells of *C. albicans* were incubated with 10 µg of antibody for 16 h and plated onto SCA plates. A reduction of 79% was observed for MAb-G5, when compared with growth control (DPBS).
to be dead that were treated with MAb-G5 (Fig. 6). During the same period, amphotericin B killed 90% of the cells while irrelevant antibody treatment had 18% dead cells. Growth control (DPBS) had more than 95% cells alive. The data represents the best of the three separate experiments. A minimal inhibition of 22% was also observed for \textit{C. parapsilosis}.

\textbf{In vivo activity of MAb}

The MAb-G5 was evaluated for its ability to protect mice against experimental vaginal candidiasis. Mice in pseudo-estrous cycle were challenged with \(3 \times 10^5\) cells of \textit{C. albicans}. After 30 min, vaginal lavages were collected from a group of 5 mice and CFU was found to be \(7 \times 10^4\) cells/ml. Mice were treated locally for three days with MAb-G5 (1 mg/ml) and the CFU was evaluated before administration of antibody (Fig. 7). A reduction of 82.6\% \((P = 0.001)\) in CFU from the vaginal lavage was observed on day five when compared with the mice that were treated with DPBS. The data was analyzed for statistical significance using ANOVA on Ranks (Kruskal–Wallis Test). The difference between the median values of DPBS: MAb-G5, and Irrelevant-MAb: MAb-G5 was significant. No significant difference was found between DPBS and irrelevant-MAb treated groups.

This monoclonal antibody was also able to protect mice prophylactically against vaginal candidiasis through intravenous route. A single dose of 100 \(\mu\)l of MAb-G5 was administered intravenously 2 h before they were challenged with \textit{C. albicans}. A reduction of 83\% \((P = 0.001)\) CFU was observed on day 5 in comparison to DPBS (Fig. 8). No significant reduction was observed in the group that was treated with irrelevant-MAb. The data passed the equal variance test and ANOVA analysis proving that there was significant difference between the MAb-G5 treated group and DPBS or Irrelevant-MAb treated group. This experiment indicates that the MAb-G5 was getting transported to the vaginal lumen. In order to confirm this finding, an ELISA was performed using vaginal lavages from animals that were administered with 100 \(\mu\)l of MAb-G5 intravenously. For controls, vaginal lavage from the same animals collected a day before the injection of MAb-G5 was used. A three-fold increase in OD proved that the antibody was getting transported to vaginal lumen.

\textbf{Discussion}

In this paper a monoclonal antibody has been defined that exhibited binding with multiple proteins of
cell wall of *C. albicans*. Many cell wall proteins of *C. albicans* have been reported to be variously mannosylated and antibodies that bind with mannosyl moieties exhibit multiple banding pattern [26]. These moieties are attached to proteins through N- and O-glycosidic linkages and are highly antigenic. Likewise our MAb-G5 had its epitope in the complex structure of mannosyl moiety that resulted in multiple banding patterns on western blot (Fig. 1). Monoclonal and polyclonal antibodies raised against mannosyl moieties have been reported to be protective in animal models of candidosis [12–14,27]. These mannosyl moieties can be detached from proteins by mild oxidation with sodium periodate. Moragues *et al.* [7] have shown that this deglycosylation resulted in loss of reactivity of monoclonal antibodies that were directed against mannosyl moieties. A similar loss in the reactivity was observed in the present study when *C. albicans* cell wall proteins on blots were oxidized with sodium periodate and probed with MAb-G5 (Fig. 1). In order to further confirm this finding the carbohydrates released by oxidation were concentrated and tested in a free phase inhibition ELISA of MAb-G5. A dose-dependent saturation of MAb-G5 confirmed that the antibody has its epitope in the intricate structure of mannosyl moiety.

There are at least two antibodies, Mycograb against HSP 90 [15] and anti-idiotypic yeast killer toxin like antibody [5], and two proteins, mannose binding lectin [8] and surfactant protein D [9] that exhibit direct candidacidal activity. Interestingly all four bind with carbohydrate moieties. Our MAb-G5 was also binding with a carbohydrate moiety and therefore, it was decided to evaluate the candidacidal activity of this monoclonal antibody. The fungicidal activity was determined by exposing cells of *C. albicans* to MAb-G5 for 16 h [25] and comparing a reduction in CFU with that of a control (Fig. 4). In another experiment, the difference in the optical densities of MAb-G5 treated and untreated cells was compared (Fig. 5). We believe that the reduction observed may partly be due to fungistatic activity of monoclonal antibody. Hence, *C. albicans* cells were exposed to MAb-G5 for a short duration of 1 h and the dead and live cells were differentially stained by PI/FDA and counted on flow cytometer. Over 63% cells were found dead proving the candidacidal activity of MAb-G5 (Fig. 6).

Bromuro *et al.* [28] have recently shown that the polyclonal sera raised against heat killed *C. albicans* modestly protect animals against systemic candidiasis. But when this polyclonal serum was made devoid of anti-mannoprotein antibodies it exhibited an elevated level of protection. On the contrary Han *et al.* [27] have shown that the mice immunized with mannan-BSA conjugates were protected against a lethal challenge with *C. albicans*. They further described that at least two monoclonal antibodies against a mannotriose were protective in murine models of mucosal and systemic candidiasis [13]. These antibodies had their epitope homogenously distributed over the surface of blastoconidia [13]. Since MAB-G5 also had its epitope distributed homogenously over the surface of blastoconidia (Fig. 2) and exhibited direct candidacidal activity the protective efficacy of this antibody was evaluated in the murine model of vaginal candidiasis. The other reason for evaluation in the mucosal model was that this antibody was of IgA isotype, and the chances were very high for this antibody to get transported to the vaginal lumen and offer protection. Similar transportation of IgM antibody has been

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**Fig. 7** A group of five BALB/c female mice were challenged intravaginally with $3 \times 10^7$ cells of *Candida albicans* in 20 μl. After 24 h these were treated intravaginally with MAB-G5 in DPBS-day 1, 2 and 3. The vaginal lavages were collected on day 1, 2, 3 and 5, and CFU/ml was determined. A reduction of 82.6% was observed in the number of CFU when compared with DPBS on day five.

**Fig. 8** The monoclonal antibody-G5 was evaluated for its ability to provide protection when administered prophylactically. A group of five female BALB/c mice received 100 μg of antibody intravenously through the tail vein, 2 h before they were intravaginally challenged with $3 \times 10^7$ cells of *Candida albicans*. A reduction of 83% was observed in the number of CFU with respect to DPBS on day five.

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reported by Han et al. [13] when administered intra-
peritoneally. In the present study protection was
observed in mice that were either treated locally with
MAb-G5 or received it prophylactically before the
challenge. These findings support the view that
although polyclonal sera raised against C. albicans
might not be protective but antibodies directed against
specific epitope may protect/treat candidal infection
[29]. In conclusion, since these results with monoclonal
IgA are encouraging a number of such fusions and
identification of such MAbS may lead to discovery of a
novel tool for treatment of mucosal candidiasis.

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