Results
Results

Isolation and storage of cell wall of \textit{C. albicans}

Cultures of \textit{Candida albicans} were maintained by routine subculture on SDA slant at 28°C. For isolation of cell wall, various methods for breaking cells like multiple cycles of freeze and thaw, sonication, grinding in presence of liquid nitrogen, vortexing with glass beads on a common vortex mixture, and vortexing in Beadbeater® were tried. Satisfactory cell breakage was obtained only by Beadbeater®. The lysate was observed under phase contrast microscope and no cell was found intact after 10-12 cycles of beating in Beadbeater®. A part of this lysate was also spread on SDA plate. No colonies were observed on this plate after 48 h of incubation at 28°C. The cell wall isolated by centrifugation was stored at -80°C. No degradation of protein was noticed when stored at -80°C. The samples that were stored at -20°C or at 4°C for duration of 2-24 h resulted in considerable degradation.

Collection of \textit{Candida} positive sera

In order to identify proteins that are immunogenic in humans, sera of patients suspected of candidiasis were collected from local hospitals and infection was confirmed by ELISA. At the same time sera of apparently normal population were also collected that served as control in ELISA and Western blotting experiments (Fig 1).

![Fig 1. ELISA titer in sera of apparently normal individuals and patients suspected of candidiasis against cell wall proteins of \textit{C. albicans}.](image-url)
Results

A clear-cut difference in the titer of patients and that of normal population was observed. Based on these data a dilution of 8000 was considered as a cutoff point and the sera falling above this value were pooled and used for identification of immunogenic proteins on 2D blots. Similarly ELISA of sera obtained from rabbit immunized with cell wall antigen of \textit{C. albicans} was performed. The zero hour sera served as control in this experiment. A titer of 64000 was observed after three booster doses of antigen in rabbit.

\textbf{Isoelectric focussing}

Two different sample buffers were used to solublize proteins of cell wall of \textit{C. albicans}. The solublized proteins were first separated by isoelectric focusing under reducing or non-reducing condition. The 2\textsuperscript{nd} dimension SDS-PAGE separation was carried out in 12\% acrylamide gel according to the method of Laemmli \cite{148}. The gels were stained with Sypro orange, Coomassie blue, or silver stained. The stained gels were scanned on regular document scanner and the images were analyzed on Seg2Dgel-2D gel electrophoresis spot segmenter program (http://open2dprot.sourceforge.net/Seg2Dgel) or Phoretix 2D gel analysis software. The data was exported to Microsoft excel and analyzed for statistical relevance.

Best results were obtained with readymade sample buffer (Sigma) under non-reducing conditions (Fig 2). A total of 1152 protein spots were detected on this gel compared to 783 spots under reducing condition (Fig 3).

\textbf{Identification of immunogenic protein spots on 2D gels}

The pooled \textit{Candida} positive patient sera were used for identification of immunogenic proteins of cell wall of \textit{C. albicans}. Proteins of cell wall separated on 2D gels and transferred onto nitrocellulose membrane were probed with pooled patient sera. A total of 38 protein spots were detected on blots. The immunogenic protein spots were assigned on gel by overlapping images of blots over that of gels and the immunogenic proteins were annotated on gels using Adobe Photoshop (Fig 4). No protein was found immunogenic on blots where the
Fig 2. Proteins of cell wall of *C. albicans* were extracted in readymade sample buffer (Sigma) and reduced with tri butyl phosphine followed by alkylation with IAA. 100 μg of protein was loaded on 10 cm tube gel. 2nd dimension run was performed in 12% isocratic gel and silver stained. The lower panel is the segmented image generated by 2D gel analysis software (Seg2Dgel [http://open2dprot.sourceforge.net/Seg2Dgel](http://open2dprot.sourceforge.net/Seg2Dgel)). A total of 1152 protein spots were counted by the software and a representative image of spots is presented in lower panel.
Fig 3. Cell wall of *C. albicans* was isolated and suspended in sample buffer. The disulfide bonds were reduced by 50 mM dithiothretol (final concentration in sample buffer) and 1.2 mg of protein was loaded on 10 cm isoelectric focusing tube gel. SDS-PAGE was performed in 12% isocratic gels and stained with Coomassie R 250 (Sigma, USA). On this gel a total of 783 individual protein spots were identified.
Fig 4. Proteins of cell wall of *C. albicans* were separated on a pH 3-8 carrier ampholytes 1st dimension IEF gels and further separated on 12% isocratic gels. One such gel was silver stained (upper panel) and another gel that was similarly run was transblotted onto nitrocellulose membrane. After blocking the membrane, it was probed with pooled human *Candida*-positive sera and developed with DAB. The image presented is the grey scale image.
cell wall proteins with their pl in basic range were resolved by NEPHGE and transferred onto NCP.

Immunoblots were also performed using sera raised in rabbit against cell wall proteins of C. albicans. Interestingly, more protein spots were observed on blots than on silver stained gel alone. This blotting helped in unambiguous identification of immunogenic protein spots on gels that were identified using pooled human patient sera. This also helped in identifying the regions of the gels where no protein spot was visible by silver staining but a protein spot was identified by both the sera.

Characterization of proteins by MALDI-TOF

Immunogenic protein spots detected on 2-D gels were excised, de-stained, and subjected to trypsin digestion. After an overnight digestion the generated peptides were collected, concentrated, and analyzed by MALDI-TOF (Micromass, USA). The spectra obtained were manually cured for irrelevant peaks and the mass values were used to search for protein using software available on internet. The 38 protein spots corresponded to 20 proteins as listed in Table 1, and their location on 2D gel is given in Fig 4.

Generation and selection of monoclonal antibodies

Spleen cells obtained from immunized mice were used for fusion with Sp2/0. Four days after fusion the culture plates were observed under microscope and the well with growing hybridoma were marked. On day six, the screening for Candida-specific monoclonal antibody producing lines was started, and continued till day eleven. During this period, more wells exhibited growth of hybridomas and were included in screening experiment. From this first round of screening over 200 clones were identified that produced monoclonal antibodies against cell wall proteins of C. albicans. These clones were further subcloned and re-examined by ELISA. Many clones were found to loose their antibody producing capacity. After 3-4 rounds of sub-culturing, and confirming on ELISA, 81 clones were left that were consistently producing monoclonal antibodies. The
Table 1. Immunogenic proteins of *C. albicans* identified by MALDI-TOF

<table>
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<tr>
<th>S. No</th>
<th>Spot No on gel image</th>
<th>Protein Name</th>
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Note: List of immunogenic proteins of cell wall of *C. albicans* separated on 2-D gels and probed with pooled human patient sera. Protein names and accession number are according to *C. albicans* genomic database (CandidaDB) from the Pasteur Institute (http://genolist.pasteur.fr/CandidaDB). The proteins reported as new, failed to correspond to any known protein present in this database.

clones that were growing but not producing monoclonal antibodies were discarded. The selected clones were further subcultured for 4-5 generations and again checked for monoclonal antibody production by ELISA. A substantial number of clones either had very low capacity of antibody production or completely lost it. All such clones were discarded and the remaining clones were propagated in 24 well culture plates that allowed collection of enough supernatant to perform Western blot against cell wall proteins separated by SDS-
Results

PAGE. All the remaining clones were found to bind with different protein bands on Western blot. Antibodies from one of the hybridoma lines were found to bind with multiple proteins on Western blots. In order to rule-out the possibility of presence of more than one clone in these hybridoma lines, the cells were again serially diluted and colonies arising from single cell were picked up, subcultured and tested by Western blotting. Western blot using pooled patient sera was also performed in parallel and the clones showing bands common with any of protein bands identified by patient sera were selected for further studies. In all 5 such clones designated as G1, MAb-G5, NE5, F3SP, and C1DP were selected for further studies.

All these five clones were slowly acclimatized to grow in serum free media. All clones adapted well in serum free media and consistently produced monoclonal antibodies. Monoclonal antibodies produced in serum free media were used in all further experiments.

**Isotype of monoclonal antibodies**

The isotype of monoclonal antibodies was determined using Immunotype kit (Sigma) as described by the manufacturer. The isotype of monoclonal antibodies (Fig 5) were identified as follows:

- G1: IgG2A
- MAb-G5: IgA
- NE5: IgM
- F3SP: IgG1
- C1DP: IgM

**Cross reactivity with other species of fungi**

The above five monoclonal antibodies developed against cell wall antigens of *C. albicans* ATCC 10231 were tested for their ability to cross react with antigens of other fungi namely *C. albicans* ATCC 14053, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 66031, *Aspergillus fumigatus*, and *Trichophyton mentagrophytes*. G1, G5 and F3SP did
not cross react with antigens of fungi other than C. albicans. NE5 and C1DP cross-reacted with all Candida species tested but not with any other yeast or mycelial fungi studied (Fig 6). NE5 was found to bind with single protein band of C. albicans but with C. parapsilosis and C. krusei, it was found to bind with two protein bands (Fig 6). Western blot of C1DP with antigens of C. albicans exhibited two bands on immunoblot, while with C. parapsilosis and C. krusei it was found to bind with a large number of protein bands (Fig 6) producing a smear.

Epitope localization on the surface of C. albicans

All antibodies were found to opsonize cell of C. albicans, although a growth state specific difference in the presence of antigen on cell surface of C. albicans was observed. In order to compare the binding of monoclonal antibodies with yeast and hyphal forms of C. albicans, culture where both the states were present was used. Epitope of G1 was localized evenly on the surface of both, the yeast and the hyphal forms (Fig 7 a and b) whereas a difference in expression of epitopes of other monoclonals was observed. The epitope of MAb-G5 was found to be evenly present on yeast forms, but on hyphal forms, the epitope was present in patches (Fig 7 c and d). The distribution of epitope of NE5 was patchy on both forms of this fungus (Fig 7 e and f). It is well known that when the yeast of C. albicans grow, the cell wall undergo extensive remodeling, and some antigens that are present at high concentration on yeast form get diluted as the fungus enters yeast-hyphal morpho-genetic transformation. This phenomenon was beautifully observed for the epitope of F3SP where a reduction in antigen aggregation was observed on the surface of germinated blastospores (Fig 8 a and b). In this figure, dense localization of antigen on the surface of yeast-bud is also clearly visible, indicating that the antigen selectively migrates on the bud during cell division. The presence of epitope of C1DP was even all over the cell surface, but the amount of antigen present on the surface of yeast form was found to be less (Fig 8. c and d). On hyphal forms, the antigen seems to dilute out completely resulting in complete absence of fluorescence on grown hyphae.
**Fig 5.** Isotype of monoclonal antibodies; G1 = IgG1, G5 = IgA, NE5 = M, F3SP = G1 and C1DP = M.

**Fig 6.** Cross reactivity of NE5 and C1DP. Numbers on the right represent molecular wt marker (kDa) and the arrows represent band positions. Ca = *Candida albicans*, Cp = *C. parapsilosis*, Cn = *Cryptococcus neoformans*, Ck = *C. Krusei*, Af = *A. fumigatus*, Tm = *T. mentagrophytes*, and Mr = molecular weight marker.
Fig 7. Epitope localization of G1, MAb-G5, and NE5 (a, c and e) using FITC-conjugated antimouse antibody and the differential interference contrast (DIC) images of the same fields (b, d and f).
Fig 8. Immuno-localization of epitope of: (a) F3SP, (c) C1DP and (e) control and DIC of the same field (b, d and f respectively).
Interestingly, the cells that burst out exhibited greater fluorescence with F3SP towards the inner side of the cell indicating that the antigen is present in greater concentrations inside the cell. Control antibodies used in these experiments did not show any fluorescence (Fig 8 e and f). No irrelevant binding of secondary antibodies was observed.

**Inhibition of attachment with composite material**

Cells of *C. albicans* are known to stick to surfaces of different substance like dental enamel, surfaces of prosthetic devices, and other composite materials used in implants including plastics/polystyrenes. Antibodies that opsonize cells of *C. albicans* reduce or abrogate this binding. An assay was performed to evaluate the capacity of monoclonal antibodies to inhibit the binding of *C. albicans* to plastic culture plates. All antibodies exhibited different levels of inhibition where monoclonals MAb-G5 and NE5 exhibited appreciable inhibition of attachment (Fig 9).

![Graph showing inhibition of attachment with composite material](image)

**Fig 9.** Exponentially growing cells of *C. albicans* were washed in DPBS and mixed with equal amount of antibody solution (1 mg/ml) such that the final concentration was $2 \times 10^3$ cells/ml. 100 µl of this was added to ELISA plate (non treated) and kept in incubator for 1 h. The wells were gently washed thrice with DPBS and cells sticking to the wells were counted under phase-contrast inverted microscope.
Inhibition of germination of *Candida* cells

Germination of *Candida*-blastospore and formation of hyphae are considered as one of the most important virulence traits of this fungus. Antibodies (monoclonal or polyclonal) of different specificities are known to inhibit this transformation to different extent by unknown mechanisms. In order to evaluate inhibition of germination by the monoclonal antibodies, cells of *C. albicans* were incubated with monoclonal antibodies in RPMI 1640 supplemented with 10% FBS at 37°C, 5% CO₂, for 4 hr. During this period, G1 and MAb-G5 reduced filamentation to 40% as compared to control, while NE5, F3SP, and C1DP completely inhibited yeast-hyphal morphogenetic transformation.

Effect of monoclonal antibodies on colony forming units (CFU)

Since the monoclonal antibodies under investigation were opsonizing and inhibited germination of blastospore, there were fair chances that the antibody might also exhibit effect on CFU. In order to determine the effect of these monoclonal antibodies on CFU, the cells of *C. albicans* were incubated with monoclonal antibodies and the CFU was determined. On comparison with control (DPBS) cent percent reduction was observed for NE5, F3SP, and C1DP, while G1 and MAb-G5 exhibited 61% and 79% reduction in CFU respectively.

MTT assay

Fungicidal activity of monoclonal antibodies was determined by MTT reduction assay where a maximum reduction of 86.3% in viability was observed for MAb-G5 when compared with DPBS. Monoclonal antibodies G1, MAb-G5, F3SP, and C1DP also exhibited comparable cidal activities. A minimal inhibition of 11% and 28.4% was also observed for the controls, Sp2/0 and irrelevant monoclonal antibody, respectively. Figure 10 represents average data from one of the three experiments performed in triplicate.
Results

Fig 10. MTT assay; Cells of *C. albicans* were incubated for 16 h, in presence of monoclonal antibodies. After incubation, MTT was added and incubated for another 1 h. MTT was converted to an insoluble formazan product that was solublized by addition of DMSO and optical density was read at 550 nm. OD value of DPBS (control treatment) was considered as 100% growth.

**FACS analysis**

In order to confirm the candidacidal activity of monoclonal antibodies, the cells of *C. albicans* were incubated in presence of mAbs for 1 h, and stained with propidium iodide (PI) and fluorescein diacetate (FDA). 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 dead that were treated with MAb-G5 (Fig 11). Variable candidacidal activity was observed for other monoclonal antibodies. 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. Data represents the best of the three separate experiments.
Fig 11. Exponentially growing cells of *C. albicans* were exposed to different monoclonal antibodies (10 μg/ml) for duration of 1 h and differentially stained for live and dead cells and analyzed on flow cytometer. On horizontal axis FITC was plotted and the vertical axis represents PI. First, the instrument settings were adjusted with unstained cells (a) and then with control (DPBS) cells stained with PI/FDA (b) where more than 95% cells were found alive. Later cells exposed to G1 (c), MAb-G5 (d), NE5 (e), C1DP (f), F3SP (g), and cells exposed amphotericin B (h) was enumerated for live and dead cells under same settings. A total reduction of 22, 63, 56, 54, 40 and 90% was observed in the number of live cells exposed for 1 h to G1, MAb-G5, NE5, C1DP, F3SP and amphotericin B respectively.
Identification of proteins to which monoclonal binds

Proteins of C. albicans cell wall separated by 2D gel electrophoresis and transferred onto nitrocellulose membrane were probed with monoclonal antibodies. The monoclonal antibody G1 resulted in appearance of a single spot on Western blot, while NE5 resulted in a train of six spots. The antigen of G1 was identified to be Hsp90 while that of NE5 as glyceraldehydes-3-phosphate dehydrogenase (GAPDH) by peptide mass finger printing using MALDI-TOF followed by database searches. Both these proteins are immunogenic in humans. Western blotting with MAb-G5 resulted in appearance of many protein spots while the other two monoclonal antibodies F3SP and C1DP were negative on blots (Fig 12).

Epitope 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 deglycosylation 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. Not all the protein bands that were recognized by MAb-G5 reacted with Con A peroxidase. In order to verify the presence of carbohydrate moiety in these bands, biotinylation of glycosylated proteins on blots was performed and detected with peroxidase-conjugated streptavidin. Another antibody NE5 used as control exhibited binding with C. albicans protein even after deglycosylation indicated that sodium periodate treatment does not degrade proteins. This was further confirmed by Ponceau S staining of the oxidized proteins on blots that did not exhibit any variation in the banding pattern (Fig 13).
Fig 12. Cell wall proteins of *C. albicans* were separated on 2-D gels and electrotransferred onto nitrocellulose membrane and stained with Ponsceau S (a). The same blot was probed with monoclonal antibody NE5 (b) that resulted in a train of six spots. Equivalent spots from a gel that was similarly run were excised and digested with trypsin. The peptides that were formed as a result of overnight digestion were subjected to peptide mass fingerprinting on MALDI-TOF. The protein was identified to be glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Similarly, the spot identified by monoclonal antibody G1 was identified to be Hsp90 (c).
Fig 13. Proteins of cell wall of *C. albicans* separated on 12% acrylamide gels were electro-transferred onto nitrocellulose membrane and stained with Ponceau S (1). The same strip was destained and probed with MAb-G5 (3). For control, another monoclonal antibody, NE5, was similarly used as probe (5). Glycoprotein detection on blots was performed by biotin-hydrazide labeling of glycosylated proteins and probed with peroxidase-conjugated streptavidin (7). Glycoprotein detection was also performed with peroxidase-conjugated concanavalin A (8).

In another set of experiment, the proteins on blots were deglycosylated by oxidation with sodium meta periodate and stained with Ponceau S (2) or probed with MAb-G5 (4) or NE5 (6) or peroxidase-conjugated concanavalin A (9).
Results

To confirm these results further, a free phase inhibition ELISA was performed. Proteins extracted from the cell wall of *C. albicans* were deglycosylated followed by extensive dialysis against diluted PBS containing protease inhibitors and the intactness of protein was confirmed by running the protein on 12% acrylamide gel. The protein value of the deglycosylated and control antigen was adjusted to 1 mg/ml and used in a free phase inhibition ELISA of MAb-G5. A dose dependent inhibition was observed with the control sample but little or no inhibition was obtained with deglycosylated protein (Fig 14).

![Graph showing free phase inhibition ELISA results](image)

**Fig 14.** Proteins of cell wall of *C. albicans* were extracted and deglycosylated by mild periodate treatment (●). These and control samples (■) were then used as free phase inhibitors of MAb-G5 in an ELISA performed against cell wall proteins.

**Epitope of C1DP and F3SP**

Proteins separated on 2D gels were electroblotted and probed with F3SP or C1DP. Repeated attempts failed to identify any protein on 2D immunoblots. Although the proteins separated by one-dimensional SDS-PAGE resulted in appearance of one wide band with F3SP at the lower half of the blot and two bands, one at the top and one at the bottom of the gel with C1DP. Boiling cell wall with SDS-sample buffer resulted in release of non-protein substances.
including mannans [102]. Thus, in search of epitope of C1DP and F3SP, the cells of *C. albicans* were autoclaved for 90 min in distilled water that released glucan and mannan from the cells with very little protein and the released components in the supernatant were fractionated into acidic glucan, mannan, and neutral glycan. The mannan fraction was further acid-hydrolyzed and the portion stable to acid hydrolysis was isolated. Of these, the mannan and neutral glucan was solublized in SDS sample buffer and run on 12% acrylamide gels, followed by transblotting and probing with C1DP and F3SP (Fig 15). The specificities of the two antibodies were also determined by dot-blot assay. The monoclonal antibody C1DP was found to bind with the acidic and neutral glucan while F3SP was reactive with the neutral glucan alone.

![Fig 15.](image)

**Fig 15.** Mannan and acidic glucan from the cell of *C. albicans* were fractionated and run on SDS-PAGE followed by electro-transfer on nitrocellulose membrane and probed with C1DP or F3SP.

**Protection against systemic challenge by passive transfer of monoclonal antibodies**

Since all the monoclonal antibodies were having their epitope on the surface of *C. albicans* cells and were opsonizing them and exhibiting direct candidacidal activity, therefore these monoclonal antibodies were tested for their ability to protect mice against systemic experimental candidiasis. All antibodies exhibited different levels of protection as determined by the kidney CFU load (Fig 16).
Fig 16. Monoclonal antibodies produced in serum free media were used to passively immunize female BALB/c mice through intravenous route. After 2 h these mice were challenged with $6 \times 10^3$ cells of \textit{C. albicans}. After 7 days the mice were euthanized and the kidney-CFU was determined by serial dilution and plating on SCA plates. No significant difference was observed among different monoclonal antibody groups. When this data was compared with that of control (ctrl-DPBS), the difference was found to be significant. The bar above represents standard deviation.

Fig 17. Pseudoestrous cycle was induced in mice by administration of oestradiol benzoate (500 µg) and after 3 days, challenged intravaginally with $2 \times 10^6$ cells of \textit{C. albicans}. The mice were divided into groups of at least 5 mice each and treated with monoclonal antibodies for 3 days. Vaginal lavages were collected and CFU was calculated by serially diluting and plating on SCA plates.
Protection in mucosal model of vulvovaginal candidiasis in mice (therapeutic and prophylactic)

All monoclonal antibodies were evaluated for their ability to treat mice against a vulvovaginal challenge with $2 \times 10^5$ cells/ml of C. albicans. Treatment with monoclonal antibodies was started 24 h post infection and continued for two more days with an interval of 24 h. Vaginal lavages were collected on 5th day and the CFU was determined by serial dilution and plating on SCA plates (Fig 17).