Generation of monoclonal antibodies against covalently linked cell wall proteins of *Candida albicans*

Thesis

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By

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Dedicated To My Wife

Acknowledgments

Tavadiyam vastu govind tubhyamev samarpaye

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(Rohitashw Kumar)

Contents

Abbreviations

AEBSF	4-(2-Aminoethyl) benzene sulfonyl fluoride
AMB	Amphotericin B
APS	Ammonium persulfate
BALB/c	Braggs albino mice
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
DAB	3, 3'-diaminobenzidine
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
GTP	Guanidine tri-phostphate
h	hour(s)
HAT	Hypoxanthine, aminopterin and thymidine
HCl	Hydrochloric acid
HRP	Horse radish peroxidase enzyme
HSP	Heat shock protein
HT	Hypoxanthine and thymidine
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilo Dalton
m/z	mass to charge ratio
MAb	Monoclonal antibody
MTT	3-(4, 5-Dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide
MES	2-(Nmorpholino) ethanesulphonic acid
NAG	N-acetyl-D-glucosamine
OD	Optical density
OPD	O-phenylene diamine dihydrochloride
PAGE	Polyacrylamide gel electrophoresis

PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PI	Protease inhibitors
PMSF	Phenyl methyl sulfonyl fluoride
PVDF	Polyvinylidene fluoride
rpm	rotations per minute
RT	Room temperature
SDB	Sabouraud's dextrose broth
SDS	Sodium dodecyl sulphate
Sp2/O	Mouse myeloma cell line
TDW	Triple distilled water
TEMED	N, N, N', N' –tetramethylethylenediamine
TLR	Toll like receptor
TMB	3, 3', 5, 5'- tetramethyl benzidine
TNF	Tumor necrosis factor
TRIS buffer	Tris [hydroxymethyl] aminomethane
YNB	Yeast nitrogen base
β-ME	β-mercaptoethanol

Introduction

Fungal infections have been known for centuries, but their clinical relevance has not been recognized until the last century. The latter half of the twentieth century has seen enormous advancement in the field of medicine and surgery. This has led to the emergence of various forms of organ transplantation and cancer chemotherapy as essential medical treatment, automatically involving immunosupression as part of the procedure. Although many health problems have been adequately addressed in this manner, the ceaseless development in modern medicine has led to the creation of “at risk individuals”, who are extremely susceptible to infections. The increasing number of immunodeficient individuals due to rapid increase in the incidence of AIDS has resulted in an epidemic of diseases caused by opportunistic fungal pathogens. Fungal infections are classified broadly into four groups: invasive infections, life-threatening infections (e.g. aspergillosis and candidiasis), mucosal infections, skin and allergic infections. The members of the genus *Candida* are known to be involved in all these four groups of diseases which are commonly known as candidiasis.

The genus *Candida* belongs to the phylum Ascomycetes that has approximately 200 species. So far, 13 species are considered to be pathogenic, of which *Candida albicans* is the most pathogenic and the most frequently encountered fungus in clinical specimens (1, 2). The main reason for this relies on its common niche, as this fungus inhabits the human gastrointestinal and urogenital tract in a significant part of the population, where it behaves as a harmless commensal organism (2). However, upon alteration of the host defences, *C. albicans* disseminates within the human body gaining access to internal organs and causing severe infections (called candidiasis) and appears as an opportunistic pathogen. The ability of this fungus to change its morphology from a yeast-like (unicellular) to a filamentous (hyphal and pseudohyphae) form (a property called polymorphism) is regulated by environmental factors, such as the temperature, the pH or the availability of nutrients, and plays a major, albeit non-exclusive role in its ability to produce disease (3, 4, 5, 6). While the biology (life cycle, metabolism and morphogenesis) of all these *Candida*

species greatly differs, they also share certain common features that enable a successful colonization of the human host and are able to counteract its defence mechanisms. Such features are frequently called virulence factors that include metabolic, structural and morphological features (7). Among others, it has been proposed that only those involved in the direct interaction with host cells should be considered as true virulence factors (8). Identification of virulence factors is an active area of research as they may provide the basis for the development of novel therapies to treat fungal infections (9, 10). While several virulence traits have been identified in many fungal species, the cell wall is still the most promising target in drug discovery for different reasons. First, it is unique to the fungus, and therefore, fulfils a basic requirement of selectivity for drug discovery. Second, it is an essential structure to the cell, whose inhibition leads to cell death (most frequently due to cell lysis). Third, and most importantly, it is the most external structure present in the fungal cell and therefore, mediates the interaction of the fungus with the mammalian host cells. As a consequence, it is involved in adhesion, colonization, signalling and immune recognition, and therefore plays a major role during infection (11).

There are a number of antifungal agents available in the market to combat the fungal infections and most of them are having fungistatic effect barring amphotericin B which is a cidal drug. Moreover these antifungals are not free from side effects and the situation is further complicated due to emergence of resistance among the patients. Most commonly used antifungal agents like azoles, allylamines and morpholines inhibit the ergosterol biosynthetic pathway. Others, such as polyenes and echinocandins, impair membrane barrier function and glucan synthesis respectively (12). The incidences of *C. albicans* cells acquiring resistance to antifungal agents like azoles and its derivatives has increased considerably in recent years which have posed serious problems towards its successful chemotherapy, probably due to widespread use of azole drugs in cancer and AIDS patients suffering from secondary fungal infections (13, 14, 15, 16). Amphotericin B, a fungicidal drug in nature has also been described to pose the problem of resistance among some *Candida* species and various fungi including several *Aspergillus* species

(15, 16). To overcome these problems, new antifungal agents targeting *C. albicans* and other opportunistic pathogens are urgently needed. Therefore, it is essential to dissect the infectious process of *C. albicans* to explore new target molecules and to search new effective, safe and broad spectrum antifungal strategies.

Production of antibodies against fungal antigens/molecules is one of the significant outcomes of the host/fungal interaction, which are important contributors to host immunity during the fungal infections. Recently, a number of monoclonal antibodies have been developed for therapeutic use against fungal infections including candidiasis for effective and safe treatment. Two monoclonal antibody based therapeutics are under clinical evaluation for treatment of fungal infections. Antibodies offer protection against candidiasis by a variety of mechanisms like opsonisation-mediated phagocytosis, inhibition of germ tube formation, inhibition of attachment of pathogen with host tissue, and direct candidacidal activity (17, 18). The development of new technologies, such as hybridoma technology for generation of monoclonal antibodies, antibody engineering and proteomic techniques that facilitated the identification of target molecules present in complex proteome, have led to a renewed interest in the study of antibody responses against fungal infections. Now a days, monoclonal antibodies are frequently used in rapid identification of *C. albicans* and other related species, characterization of target molecules /epitopes in the process of cell wall assembly and in diagnosis of candidiasis by detection of circulating antigens in patients (19, 20).

Cell wall is the outermost cellular structure, which determines the shape of fungal cell. Cell wall remodelling is required for the morphological conversion of *C. albicans* from the yeast to the filamentous form, which involves alteration in its composition and organization. It indicates plastic and dynamic nature of the cell wall, which changes constantly in response to environmental signals, and the different stages of the fungal cell cycle. Because of its privileged location within the cell, cell wall is also the initial point of contact between the cell and the environment and thus contributes to host fungus interaction. In addition given that mammalian cells lack a cell wall, this cellular compartment

could be a promising molecular site to search for new specific antifungal drug targets. Further, the cell wall proteins play a key role in morphogenesis and pathogenesis (21, 22, 23). The cell wall of *Candida albicans* consists of an internal skeletal layer and an external protein envelop. This layer has a mosaic-like nature, containing approximately 20 different protein species covalently linked to the skeletal layer. Most of them are glycosylphosphatidylinositol (GPI) proteins. Covalently linked cell wall proteins vary widely in function and many of them are involved in the primary interactions between *C. albicans* and the host and mediate adhesive steps or invasion of host cells. Others are involved in biofilm formation and cell-cell aggregation. An important role in the fitness and virulence of *C. albicans* is reserved for those cell wall proteins that are covalently linked to the skeletal cell wall polysaccharides. Covalently linked cell wall proteins play a crucial role in the capability of *C. albicans* to survive and grow in the host and to cope with the stress conditions associated with the host infections (24, 25).

The main objective of the present work was to generate monoclonal antibodies against covalently linked proteins of *C. albicans* cell wall and to evaluate their therapeutic and/or diagnostic value for candidiasis. In this piece of work, isolation and characterization of covalently linked proteins from cell wall of both yeast and hyphae of *C. albicans* was done. The covalently linked cell wall proteins were characterized by peptide mass fingerprinting using MALDI-TOF-MS technique. As GPI cell wall proteins consist of largest fraction in covalently linked proteins, hyphal GPI cell wall proteins were used for generation and evaluation of monoclonal antibodies for their therapeutic and diagnostic potential *in vitro* and also against *in vivo* model of murine candidiasis. Paratope derived ppeptides were designed from the sequences obtained by reverse transcription and cDNA sequencing of hybridoma line showing the most protective response. Along with generation of monoclonal antibodies, amphotericin B resistant strain of *C. albicans* was developed and characterized on the basis of various virulence factors and at genetic level too. Monoclonal antibodies generated against covalently linked cell wall proteins were also evaluated for their efficacy on amphotericin B resistant strain.

Review of Literature

The frequency of human fungal infections has increased significantly over the past 20 years. This increase in infection is associated with enhanced morbidity and mortality and is directly related to the increasing numbers of patients who are at risk for the development of serious fungal infections, including patients undergoing blood and marrow transplantation (BMT), organ transplantation, and major surgery (especially gastrointestinal surgery), patients with AIDS, neoplastic disease, and advanced age, patients receiving immunosuppressive therapy, and premature infants (1, 2, 3). The diverse population of patients who are at risk for infection and increasing array of opportunistic fungal pathogens set considerable diagnostic and therapeutic challenges. The list of opportunistic fungi causing serious, life-threatening infection increases every year. In addition to *Candida*, *Aspergillus*, and *Cryptococcus* species, the opportunistic fungi include yeasts other than *Candida* species, nondematiaceous or hyaline molds, and the pigmented or dematiaceous fungi (3, 4).

Among all the opportunistic fungal pathogens, most important group of opportunistic fungal pathogens are the *Candida* species. Candidiasis, the infection caused by the *Candida* species, is considered to be an important medical problem in both developing and developed countries. Candidiasis is predominantly an infection of the immunocompromised host which implies that for *Candida* to cause an infection; one or more risk factors should be present. A number of factors have been suggested, including underlying immune insufficiency (HIV/AIDS), use of broad-spectrum antibiotics, cytotoxic agents (e.g. anticancer agents), immunosuppressive drugs, and central venous catheters. Although the genus *Candida* comprises of almost 200 yeasts species, few species have been associated with infection to significant degree. These include: *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis, C. guilliermondii, C. pseudotropicalis, C. rugosa,* and *C. stellatoidea, out* of which *C. albicans,* which is the etiological agent of up to 75% of candidal mycoses and is the most virulent species of *Candida* (2, 3).

2.1. Clinical Manifestation of *Candida albicans* Infection

C. albicans is a commensal of the human microbiota of the gastrointestinal tract, mucous membranes, and skin. The incidences of infections caused by *C. albicans* were reported during Roman Empire era as the name has originated during that period. The term “Candida” referred to the white robes worn by members of roman senate, whereas “albicans” is a Latin word meaning “to whiten”. Thus, *Candida albicans* literally translates to “Whitening white”. This corresponds to the growth seen at the site of infection.

Candidiasis is an opportunistic fungal infection caused by *Candida* species (especially *C. albicans,* commonly represented in normal flora on human mucosal surfaces) that currently ranks among the most frequent of nosocomial diseases. It comprises a wide spectrum of diseases that range from superficial mucosal lesions (e.g. oropharyngeal, esophageal, and vulvovaginal candidiasis) to invasive or systemic forms of infection (1, 2, 3).

2.1.1. Systemic Candidiasis

Severe organ invasive or systemic hematogenously disseminated candidiasis (DC) is characterized by spreading of the *C. albicans* cells into almost the entire body with a tendency to create abscesses in vitally important organs, inducing their failure which leads to mortality in ~50% of all cases, irrespective of administration of intensive antifungal therapy (26, 27). The common clinical signs of ongoing systemic candidiasis are hyper- and/or hypothermia, tachycardia, hypotension, high white blood cell counts, left shift, the need for vasopressor (26). Individuals at risk for systemic candidiasis are those with polymorphonuclear cell (PMNs) defects, such as patients with prolonged neutropenia due to bone marrow transplant or chemotherapy (particularly for leukemia) (27). In neutropenic patients, it starts either as a contamination of indwelling catheters or translocation of endogenous *C. albicans* from the GI tract. In these cases previous colonization by *C. albicans* is also a risk for candidemia (*Candida* in the blood). Once *C. albicans* has reached the bloodstream, virtually any organ or body site can be infected. Systemic infections are fatal if left untreated, and, even when patients are receiving the

appropriate antifungal therapy, the mortality rate is as high as thirty-five percent (26). Furthermore, there are no specific diagnostic tests for the rapid detection of systemic candidiasis (28, 29, 30).

2.1.2. Oral Candidiasis

C. albicans is a commensal organism of the mouth, where carriage rates in the general asymptomatic population range from 20% to 75%. Oral candidiasis/oropharyngeal candidiasis (OPC) is the most common human fungal infection, especially during child hood and old age because of reduced immunity. Populations most susceptible to oropharyngeal candidiasis (OPC) are individuals with congenital or acquired immunodeficiency (particularly T cell deficiencies), individuals using broad-spectrum antibiotics, patients who have leukaemia or are undergoing chemotherapy, neonates, diabetics, smokers, denture wearers, and patients who are terminally ill. OPC is also the most common infectious complication of patients with acquired immunodeficiency syndrome (AIDS), and it is well recognized as both an indicator of HIV infection and a predictor for AIDS progression (32). Other factors that predispose to OPC are impaired salivary gland function, inhaled steroids, dentures, and oral cancer. Saliva, by means of its secretion, not only removes organisms from the mucosa but also contains antimicrobial proteins, such as lactoferrin, sialoperoxidase, lysozyme, histidine-rich polypeptides, and specific anti-*Candida* antibodies that prevent overgrowth of *Candida*. Inhaled steroids can also suppress cellular immunity and phagocytosis locally, which is required to keep commensal organisms in check. Dentures predispose one to OPC due to the reduced flow of saliva under surfaces of the denture, the propensity for *Candida* to adhere to acrylic, and the general state of poor oral hygiene that often accompanies the use of dentures (33).

2.1.3. Vulvovaginal Candidiasis

C. albicans is the main cause of vulvovaginal candidiasis (VVC), which affects a significant number of women. It is known that up to 75% of all women will experience at least one episode of VVC in their life span. Predisposing factors are antibiotic and oral contraceptive usage, hormone replacement therapy,

pregnancy, uncontrolled diabetes mellitus, immunosuppressive therapy, and possibly HIV infection. In a small subset of women (5-10%) recurrent episodes of VVC can occur and it is postulated to be due to an immune deficiency (34, 35, 36).

2.2. Virulence Factors in *C. albicans*

Because of the complex nature of the host-fungus interaction, there are few factors that are absolutely required for fungal virulence. Virulence factor can be defined as a component of a pathogen that interact directly with mammalian host cells and damages the host cells for the spread of diseases. *C. albicans* is not only a versatile organism that is able to persist at different anatomical sites but also a successful pathogen that adapts to a range of environments exerted at different sites within the host (22, 37, 38). Pathogenesis of *C. albicans* has been attributed to the expression of a variety of virulence factors, which allow it to adhere to host tissues, survive and grow at different body sites, cause damage, evade host immunity, and spread to other tissues (39).

2.2.1. Adhesion Factors

The adherence to the host cells and tissues, as well as the binding of a set of diverse host proteins is essential for *C. albicans* to begin the invasion, followed by dissemination within the human organism. This step is crucial for fungal survival. Some receptors are present on the cell wall surface of *C. albicans* receptors which are responsible for adhesion to epithelial and endothelial cells, serum proteins and extracellular matrix (ECM) proteins which are known as adhesins (biomolecule that promotes the adherence of *C. albicans* to host cells or host-cell ligands) (22). The ability of *Candida* to invade different environments in the host organism is a result of great flexibility and adaptability of fungi. This phenomenon is due to the presence of different adhesions connected with cell surface, which facilitate the first stage of infection. These adhesins include ALS proteins family, Hwp1p, Eap1p, Csh1p, and other less known cell surface receptors (40).

2.2.1.1. ALS (Agglutinin-Like Sequence) Protein Family

The family of ALS proteins is one of the well-known examples of *C. albicans* adhesins. This family includes at least eight *ALS* genes that encode proteins with similar structure, which are connected to the host cell surface. Als1p, Als3p and Als5p proteins has been believed to be responsible for adherence to collagen, fibronectin, laminin, endothelial and epithelial cells, Als6p binds to collagen and Als9p to laminin and Als4p probably mediates adherence to endothelium, whereas Als5p is additionally responsible for cell-to-cell aggregation. The role of Als7p is still unknown (41, 42, 43, 44, 45, 46).

2.2.1.2. Transglutaminases (TGases) Substrate Protein Family

The well known example of transglutaminases is Hwp1, a receptor with a molecular mass of approximtely 34 kDa, which is present only on the surface of hyphal cells (22). *HWP1* encodes an outer surface mannoprotein that is believed to be oriented with its amino-terminal domain surface-exposed and the carboxyl terminus most probably covalently integrated with cell wall β-glucan and facilitates adhesion to epithelium ((47, 48). Another receptor, Eap1p shows homology and structural similarity to Hwp1p, but the host ligands for this protein are still unknown (46).

2.2.1.3. Integrin-like Protein Family

Candida albicans adhere to platelets and endothelial cells with help of different extracellular matrix components (22, 42, 49). *C. albicans* possess several proteins containing specific binding sites which are indicative to mammalian cell receptors. Extracellular matrix (ECM) components of host form a network by interactions with each other thus providing multiple binding targets to the pathogen. The receptors are expressed extensively on hyphae and this has been postulated as one of the ways by which germ tubes contribute to the development of infection (48). A putative integrin like gene *INT1* has been identified in *C. albicans* which contains an I-domain which is approximately 18% similar to human α-M integrin domain involved in the coordination of the divalent cations required to bind to the ECM proteins (50, 51).

2.2.1.4. Hydrophobic Proteins

Adhesion to host cells is also dependent on interactions between mannoproteins with lectin-like properties and fucosyl or glucosaminyl glycosides on epithelial cell surface (52). Although many fungal adhesins have been identified, til now a little is known about the host receptors involved in adhesion. A novel 38 kDa receptor, Csh1p, which enhances hydrophobicity of *C. albicans* cells and facilitates specific receptor-ligand interactions, has been identified (52). Toll-like receptors 2 and 4 and other receptors present on the surface of human immune cells, such as monocytes, macrophages and dendritic cells are involved in receptor-ligand interactions. Some hydrophobic proteins present on the surface of host epithelial or endothelial cells, such as N-cadherin, are important for binding and endocytosis of different morphological forms of *C. albicans* cells (46, 49).

2.2.2. Penetration and Dissemination Factors

Penetration of host tissue is the first step in fungal infections that may be a limited step for entry of fungal pathogen to become systemic, disseminating either via haematogenous or contiguous routes. Movement from the infecting surface into the bloodstream requires tissue damage which may be pre-existing or can occur either by mechanical penetration or new tissue necrosis. *Candida albicans* hyphae respond thigmotropically (movement toward or away from a touch stimulus) and morphologically to host tissue damage. Thus, thigmotropism may enhance the ability of hyphae to invade epithelia of a host at sites of weakened integrity or to follow vasculature (54).

Fungi may also spread from the site of infection throughout the host by such mechanisms like host phagocytosis. *C. albicans* invades endothelial cells through being phagocytosed. After binding to endothelial cells, the fungus invades these cells by inducing its own endocytosis which requires hyphal growth forms to bind to N-cadherin on the endothelial cell surface. C*. albicans* then migrates through the endothelial layer thereby damaging it (46, 53, 55). It is currently not clear how *C. albicans* can escape from endothelial cells.

2.2.3. Nutritional and Metabolic Factors

The inability of *C. albicans* to synthesize purines, pyrimidines, or heme *de novo* has shown significant reduction in its virulence (56). The ability to synthesize fatty acids has also been shown to be essential for *C. albicans* both in a systemic mouse model and in a rat model of oropharyngeal candidiasis. Null mutant or heterozygote of *FAS2* gene of *C. albicans* strain (auxotroph for several fatty acids) led to reduced virulence (57, 58).

Glycolytic enzymes represent those *C. albicans* proteins which are immunogenic during oral and systemic infections (59). Changes in glycolytic gene expression accompany the morphological transition in *C. albicans* and reflect the underlying physiological status of the cells during morphogenesis (60, 61).

2.2.4. Necrotic Factors

Necrotic factors allow the fungus to overcome structural barriers that the human host uses to prevent invasive infection. Most necrotic factors are hydrolytic enzymes which help in opportunistic nature of *C. albicans* as their major role in infection is degradation of host tissue. The secretion of hydrolytic enzymes during infections may be required as virulence attribute for the degradation of host surfaces to aid adhesion, invasion, and destruction of host immune factors, in addition to nutrient acquisition (62).

2.2.4.1. Secreted Aspartyl Proteinases

The secreted aspartyl proteinase family contains 10 genes (*SAP1-10*), which are differentially expressed during different stages of infection and morphological transition (62, 63). They facilitate adherence to many host tissues and cell types by modifying target proteins or ligands on either the fungal surface or the host cells. The production of aspartyl proteinases by hyphae allows it to penetrate and invade tissues by digesting and distorting host cell membranes. *SAP2* can hydrolyze many humoral host defense proteins, such as secretory IgA, thus allowing the yeast to evade the host immune system. *SAP4, 5,* and *6* have been shown to be up-regulated in

hyphal cells and are required for the invasion of the liver and the pancreas in a murine intra-peritoneal infection model (62). *SAP5* in particular was up-regulated in the liver and kidneys in this model and in the kidneys in an intra-venous model. The contribution of Sap hydrolytic activity to tissue damage and invasion has been confirmed by the addition of pepstatin A, an aspartyl proteinase inhibitor. Treatment of mice with pepstatin A prior to and during *C. albicans* infection reduced mortality in intra-nasal and intra-peritoneal models but not in an intra-venous model of infection (64). Laboratory strains and clinical isolates of *C. albicans* exhibited direct correlation of virulence with a number of *SAP* genes and levels of proteinase production (65, 66). More virulent strains of *C. albicans* have been isolated from patients with AIDS than from normal subjects, and this characteristic may be associated with an elevated production of aspartyl proteinase (67, 68). Sap proteins have been reported to be rare or absent in non-pathogenic strains or species of *Candida* and mutant strains of *C. albicans* that do not secrete this enzyme show significant reductions in lethality in mouse model. It has also been suggested that the proteinases could promote the release of cell wall mannan by cleaving the peptide moiety of candidal cell wall mannoproteins. The released mannan may cause stimulation or suppression of cell-mediated and humoral immune functions. The enzyme is able to degrade a number of important defensive host proteins such as immunoglobulin's and complement (69, 70). Sap proteins hydrolyzes mucin *in vitro*, which suggests a role in penetration of the gastrointestinal mucin barrier and providing an access to underlying cells (22).

2.2.4.2. Phospholipases

Three phospholipases, phospholipase A, B, and C of *C. albicans* have been found associated more closely with the walls of older yeast cells than that of younger cells and were more prominent at the tip of growing hyphae than in the lateral walls (71, 72, 73). Phospholipases are associated with membrane damage of the host cells, adherence, and penetration. Ibrahim *et al.* (71) showed that blood isolates of *C. albicans* showed higher phospholipase activity compared to their commensal counterparts. They also showed that

strains with higher phospholipase activity were more invasive in mouse model than those with low phospholipase activity and level of phospholipase activity is more reliable in the prediction of mortality compared to other virulent factors. Phospholipids are major components of biological membranes and membrane-bound vesicles and contribute to their structure and function. Among the natural phospholipids, phosphatidylcholine (PC, lecithin) is most common and a suitable substrate for phospholipase followed by phosphatidylinositol, phosphatidylserin and phosphatidyiinositol. Arachidonic acid, a 20 carbon containing unsaturated fatty acid, is a constituent of these phospholipids linked through ester bond. Phospholipase produced by *C. albicans* results in the release of AA by breaking ester bond. AA is converted to 3-HETE [3(R)-hydroxy-5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid] which acts as a growth factor for the fungus (74).

2.2.4.3. Lipases

Lipases are a gene family (*LIP1-LIP10*) that have been shown to be differentially expressed at different stages and sites of infection, including a systemic mouse infection model (75, 76). Although some of the *LIP* genes (*LIP1, 3–6, 8*) were expressed during growth on lipids as the sole carbon source, these were also expressed in the absence of lipids (72). *LIP2* and *LIP9* were only expressed in the absence of lipids, suggesting an alternative role to nutrient acquisition for these lipases. As yet there have been no reports describing the effect of deleting the *LIP* genes to establish a possible role in virulence. However, this may be a complicated process due to the similarity of the members of this gene family to the large number of genes.

2.2.5. Morphological Factors

2.2.5.1. Morphogenesis

C. albicans is a polymorphic fungus exhibiting reversible morphogenetic transitions between budding unicellular yeast form (blastospores), pseudohyphal, and hyphal forms (11, 77). Yeast cells are oval to oblong cells often seen with daughter cells still attached to the mother cell in the form of a bud. The pseudohyphal form ranges from relatively short to extended cells,

while the hyphal forms possess constrictions at the septa. Hyphae and germ tubes are narrower and more uniform than pseudohyphae (7). The morphological transition between these forms is considered to be necessary for virulence which enables the pathogen to colonise different loci of the host, such as oral and vaginal tracts, and to invade the parenchyma of inner organs via blood stream (11, 78). This is supported by the fact that mutant strains of *C. albicans* incapable of hyphal formation are, in general, less virulent in mouse model of disseminated or mucosal candidiasis. *C. albicans* yeast forms are rapidly eliminated from the blood in normal host. Survival of this fungus depends upon rapid conversion to hyphal form in host microenvironment. Germ tube and hyphae exhibit increased adherence which enables the cells to attach to the blood vessels and to penetrate through the endothelial layer (79). Many adhesins are found in increased levels on hyphae and germ tube. Also hyphal form produces large amounts of SAP proteins. SAP4 and SAP6 proteins are exclusively produced during dimorphic transition. In hyphal cells, surface anionic radicals are more abundant, especially at the apex, which play a role in pathogenicity and adherence (80). Hyphal cells contain at least three times more chitin than yeast cells and escapes immune system more efficiently (81).

In vitro factors that induce formation of hyphae include temperature greater than 37°C, pH greater than 6.5, high CO_2 to O_2 ratio, low inoculum ($<10^6$ blastoconidia/ml), presence of chemical factors like N-acetyl-D-glucosamine, glucose with glutamine or glucose with glycine, proline, low zinc concentration, dextrin and particular formulations of growth media which include amino acid/salt medium, neopeptone-starch medium, TC199, and MEM medium (82, 83, 84). Thus no single environmental factor is responsible for morphogenesis. *In vivo* host derived factors like prostaglandin E_2 (PGE_2), estrogen, progesterone are responsible for morphogenetic transformation in *C. albicans* (85, 86, 87).

2.2.5.2. Phenotypic Switching

Phenotypic switching is the capacity of epigenetic change (regulation of expression of gene activity without alteration of genetic structure) in colony morphology. The ability to undergo phenotypic switching helps *C. albicans* survival in different microenvironments and evasion of the immune response. In addition to morphological transitions, the yeast cells of *C. albicans* assume two distinct cell types, designated “white” and “opaque”. Each cell type is stably inherited for many generations, and switching between the two types of cells occurs stochastically and rarely (88). White form is the most commonly occurring form with dome shaped colony morphology and oval shaped cells. Opaque cells are oblong shaped with twice the size of white cells, with a large vacuole and form flat colonies. They differ in their behaviour towards other *C. albicans* cells. Opaque cells also are highly competent for mating and respond to mating pheromone with polarized growth, and can subsequently undergo cell and nuclear fusion with opaque cells of the opposite mating type (89). The opaque forms are more often found in skin infections while the white form is seen in blood infections (90, 91). Opaque cells have altered permeability and altered sterol and lipid composition and thus show altered drug sensitivity (91, 92). They also differ in their susceptibility to neutrophils and phagocytic killing (93).

2.2.6. Other Factors

Other traits have been implicated as important for the pathogenicity of *C. albicans*. Increased resistance against antimicrobial drugs help a pathogen to avoid their clearance from the host (2, 94). In *C. albicans*, resistance mechanisms include the mutation of the antifungal targets themselves and the up-regulation of the multidrug transporters Cdr1, Cdr2, and Mdr1 (95, 96). To defend against reactive oxygen species during phagocytosis, several *Candida* species have been shown to produce an antioxidant agent (2,4-(hydroxyphenyl)-ethanol), which protects them against neutrophil-mediated killing (97). In *C. albicans* the superoxide dismutase 5 (Sod 5), and even cell wall mannans, may give some protection against oxidative stress while

flavohaemoglobin provides defence against nitric oxide (98, 99). *C. albicans* also has antioxidative stress responses regulated by Hog1 and Cap1 (100, 101). In addition, the ability to assimilate iron is necessary for survival of all pathogens in the host and could hence be called a persistence factor, but is frequently considered a virulence determinant (102).

2.3. Host Defence against *Candida albicans*

In the immunocompetent host, opportunistic fungal pathogens are systemically eradicated by innate immune defense. The most important risk factor for opportunistic *Candida albicans* infection is an impaired host defence system. The nature and extent of the impairment of the host defence translates into manifestation and severity of the *Candida* infection. Protective immunity against fungal pathogens is achieved by the integration of two distinct arms of the immune system, the innate and adaptive responses. Innate and adaptive immune responses are intimately linked and controlled by sets of molecules and receptors that act to generate the most effective form of immunity for protection against fungal pathogens.

2.3.1. Innate Immunity Mechanism

Innate immunity is the dominant protective mechanism against systemic candidiasis which causes quantitative and qualitative abnormalities of neutrophils and monocytes. Neutrophils and monocytes damage and kill yeast cells, hyphae and pseudohyphae. Neutrophils and monocytes recognize and engulf opsonised and non-opsonised yeast cells via cell-surface pattern recognition receptors, including Toll like receptors (TLRs), mannose receptors and beta-glucan receptors. Binding to individual TLRs or IL-1 receptor (IL-1R) activates specialized antifungal functions on neutrophils and other phagocytes. Killing of fungal cells is by oxidative and non-oxidative mechanisms, including generation of reactive oxygen and nitrogen intermediates. Phagocytosis and killing are augmented by opsonisation and proinflammatory cytokines. Invasion of vascular structures facilitates dissemination of *Candida*. Endothelial cells resist vascular invasion by secretion of proinflammatory mediators and expression of leucocyte adhesion

molecules, which recruit and bind to activated leucocytes. Mediators of inflammation at the site of damaged endothelial surfaces induce release of antimicrobial peptides from human platelets (101, 102, 103).

2.3.1.1. Polymorphonuclears

Polymorphonuclears (PMNs) are the first line of defense against systemic candidiasis. They are short lived cells with phagocytotic and microbicidal properties. PMNs are crucial for the initiation and magnitude of cell-mediated and humoral immunity through the secretion of soluble mediators. Results have shown that killed *C. albicans* can stimulate *in vitro* PMNs to release IL-1, IL-6, and TNF-α and *Candida* mannoproteins stimulate PMNs to produce GM-CSF (103, 104, 105, 106). Vulvovaginal candidiasis (VVC) or recurrent vulvovaginal candidiasis (RVVC) inflammatory symptoms are associated with the presence of aggressive PMNs attracted to vaginal lumen by *C. albicans*. Although some *in vitro* experiments demonstrated strong *Candida*-static activity of PMNs, in vaginal environment PMNs are not able to protect successfully against *Candida* (106, 108). Thus PMNs in vaginal environment could be only harmful instead of protective during VVC or RVVC. The involvement of PMNs in protection against OPC is in preliminary stages. During OPC the PMNs ordinarily infiltrate the mucosal tissue where they are activated by local cytokines, later in the course of infection the activity of PMNs is suppressed by IL-4 (109, 110). The evidence for a protective role of PMNs arises from observation that OPC can be relatively common in neutropenic patients (109).

2.3.1.2. Macrophages

Macrophages are effector cells that initiate inflammatory responses by both oxygen and nitrogen radicals to kill fungi (110, 111, 112). The effectiveness of fungicidal activity depends on the macrophage activation status. Phagocytosis of *Candida* cells by mouse peritoneal macrophages induces rapid fusion of *Candida*-containing phagosomes with the late endosomes or lysosomes. This can trigger germ tube formation and escape of *Candida* cells and the destruction of non-activated macrophages. Subsequently, the germ tube can

penetrate surrounding intact macrophages by phagocytosis-independent manner (113). Thus phagocytosis of *Candida* by the non-activated macrophages can contribute to changes toward more pathogenic *Candida* morphotypes. The inflammatory microenvironment can activate macrophages and prevents the escape of *Candida* (112).

2.3.1.3. Natural Killer Cells

Natural killer (NK) cells are large granular lymphocytes that preferentially kill cells lacking MHC class I expression (114). NK cells bind to *C. albicans* and one of the surface structures of NK cells that mediate adhesion to *C. albicans* has been shown to be β2-integrin CD11b/CD18 (115). Interleukin IL-2 activated natural killer cells bind more extensively to *Candida* germ tubes than NK cells, but both are unable to kill or inhibit germ tube or hyphal growth forms of *C. albicans* suggesting that NK cells do not have an important direct anti-candidacidal effect (116, 117, 118, 119). Hence, NK cells play an important role in anti candidacidal host defence through activation of phagocytes. There are evidences that NK cells are the main inducers of phagocytic activity of splenic macrophages and that they mediate the protection against systemic *C. albicans* infection (120).

2.3.1.4. Epithelial Cells

Epithelial cells play an important protective role in both vaginal and oral candidiasis. Under normal circumstances, *Candida* counts are maintained in vaginal lumen with the aid of vaginal epithelial cells (VECs). The *Candida*-static activity of VEC was confirmed both in animal models (mice, macaques) and in humans (109, 121). VECs require direct contact with the *Candida* cells through a yet unknown sugar moiety other than sulfated polysaccharides, sialic acid residues, and glucose and mannose containing saccharides but they do not require signals mediated by soluble immune factors (121, 122). Changes occurring in VECs during the estrous cycle can induce an episodic increase in *Candida* counts. In some cases, this could lead to development VVC or RVVC. Increased frequency of VVC during the last trimester of pregnancy can be, at least partially, attributed to hormonal changes. Besides

additional *Candida* cells are acquired through sexual transmission during child-bearing age, typically associated with increased frequency of VVC and RVVC (107). At this point it is possible to postulate that VVC is the clinical demonstration of an ineffective effort of the activated vaginal mucosa where VEC and PMNs failed to put the *Candida* load into its previous commensal typical state.

2.3.1.5. Complement System and Mannose Binding Lectin

The complement system is a group of heat-labile proteins which can be activated in a cascade fashion to provide an innate defence against various pathogenic microbes. Complement activation is initiated via several pathways that all converge at a single point, the third complement component (C3). The role of complement in candidiasis is evidenced by several observations. First, *C. albicans* activates the complement cascade via both the classical (antibody-antigen) and alternative pathway (cell surface material with repeating chemical structure) and shows binding of C3 fragments (123, 124). Second, the presence of C3 promotes the ingestion of *C. albicans* by phagocytes and is prevented by anti-C3 and the third, C5-deficient mice show an increased amount of *Candida* colony forming units (cfu) in their organs and an increased mortality in disseminated candidiasis, probably due to lack of C5a, an important leukocyte chemoattractant (125, 126). Moreover, the classical pathway of the complement cascade can also be activated by mannose binding lectin (MBL). MBL is another important serum protein of innate immunity that is synthesized by the liver. MBL is able to recognize and bind to multiple sugar ligands expressed on the surface of microbes, such as mannose on the surface of *C. albicans* (127, 128, 129, 130). It has been shown that MBL initiated complement activation may play an important role in anticandidal host defence of previously unexposed and non-immune hosts too (127).

2.3.2. Acquired Immunity

Antigen-independent recognition of fungi by the innate immune system leads to the immediate mobilization of immune effector and regulatory mechanisms

that provide the host with the rapid initiation of the immune response and creation of the inflammatory and co-stimulatory environment for antigen recognition.It also helps in establishment of a first line of defense, which holds the pathogen in check during the maturation of the adaptive immune response and steering of the adaptive immune response towards the cellular or humoral elements that are most appropriate for protection against the specific pathogen. Receptors on phagocytes not only mediate downstream intracellular events related to clearance, but also participate in complex and disparate functions related to immunomodulation and activation of immunity, depending on cell type. Therefore, in order to achieve optimal activation of antigen-specific adaptive immunity, it is first necessary to activate the pathogen-detection mechanisms of the innate immune response (130, 131, 132).

2.3.2.1. Cell Mediated Immunity

Host cell mediated immunity is mediated by T lymphocytes, cytokines and a number of effector phagocytes. For all fungal infections, T-cell activation is a crucial element in the development of optimal protective immunity. Both CD4+ and CD8+T cells are necessary for the elimination of fungal pathogens. However, for many fungi, the presence of CD4+T cells is vital for the survival of the host in the primary stages of infection, whereas CD8+T cells are necessary to restrict infection (131, 132). The main effector mechanisms of T cells are cytotoxicity and cytokine secretion. The role of cytotoxicity in host defence against fungi is not well defined as compared to the activity of cytokines which is better understood. For all pathogenic fungi, a T_H1 response is the dominant adaptive response as the absence of the T_H1 cytokines interferon (IFN)-γ or tumour necrosis factor (TNF)-α leads to overwhelming disease. The T_H2 response is often associated with a subversion of the host response to fungi. Increases in the T_H2 cytokines IL-4 and IL-10 are commonly observed in progressive disease and neutralizing their activity restores protective immunity. One possible beneficial effect of T_H2 cytokines is that

they dampen the damage associated with an exuberant inflammatory response (133, 134, 135).

Dendritic cells play a principal role by sensing the fungal morphotypes through several surface receptors such as FcγR, complement receptor (CR), or pattern-recognition receptors (PRRs) which include mannose receptor (MR), β-glucan receptor, and Toll like receptors (133, 136). Recognition of pathogen-associated molecular pattern (PAMP) of *C. albicans* yeast through MR on the surface of the DCs leads to production of IL-12 (associated with interferon production and T_H1-type responses). Other DC-surface receptors, such as CR3 (CD11b/CD18), MR and FcγR, bind only opsonised yeasts or hyphae and the binding leads to production of IL-4 and IL-10 associated with T_H2 and Treg responses, respectively. Opsonisation of the *Candida* surface with C3, immunoglobulin (Ig), and/or mannan-binding lectin (MBL) can mask the actual *Candida* morphotype-determined PAMPs and consequently can influence the DC receptor used, thus qualitatively affecting DC activation. Opsonisation *via* C3 and IgG facilitates the uptake of yeast through CR3 and FcγR, resulting in T_H2 or Treg responses. Opsonisation *via* C3 together with MBL increases the uptake of yeast through CR3, resulting in blockade of the T_H1 responses (135, 137).

Toll like Receptors (TLRs) comprises the group of PRRs which seems to be closely morphotype specific and involved in the recognition of fungi and Gram-positive bacteria PAMPs (138). The TLR2 and TLR4 on DCs are also involved in the induction of protective T-cell memory against hyphal reinfection associated with increased level of IL-10. Moreover, TLR2, TLR4, and TLR9 on PMNs stimulated upon exposure to fungi, zymosan, lipopolysaccharide, or CpG oligonucleotides increased the fungicidal activity of PMNs (139).

2.3.2.2. Antibody Mediated Immunity

In 1987, Dromer *et al*. demonstrated that passive administration of an immunoglobulin (Ig) G1 monoclonal antibody (MAb) against the *Cryptococcus neoformans* polysaccharide could mediate protection in mice given lethal infection (140). In contrast to prior studies, which relied on polyclonal sera, the

application of hybridoma technology to fight this problem was an important step because MAbs are invariant, theoretically available in unlimited quantities, and produce consistent results in immunological studies. Experiments with MAbs revealed that antibody efficacy against *C. neoformans* were a function of isotype, dose and epitope specificity (141, 142, 143). For *C. albicans*, two IgM monoclonal antibodies were described that differed sharply in protective efficacy (144). The identification of protective and non-protective antibodies for both *C. neoformans* and *C. albicans* indicated that humoral responses to fungi could elicit antibodies of variable efficacy and suggested an explanation for the historical difficulties encountered in studies of humoral immunity (145). In the last decade, a new consensus has emerged based on the view that humoral immunity can protect against fungi provided that certain types of protective antibodies are available in sufficient quantity.

Antibodies protect the host from infectious processes by various complementary mechanisms (Figure I). The simplest example is direct interaction between the antibody and the foreign material, causing its neutralization. In other cases, additional factors that interact with antibodies include constituents of the innate immune system such as complement and cellular components, most notably neutrophils, monocytes and macrophages. The most complex mechanism, which is the least understood, involves the linking of antibody function to T-cell-dependent immunity.

In recent years additional mechanisms of antibody action against fungi have been revealed, including growth inhibition, inhibition of biofilm formation, inhibition of adherence, inhibition of germination, and direct antifungal effects (145, 146, 147, 148, 149). For antibodies against *C. albicans* mannoproteins, *Pneumocystis carinii* surface antigen, and *C. neoformans* glucuronoxylomannan, the Fc region and/or complement was essential for antibody efficacy whereas the activity of antibodies to other *C. albicans* mannoproteins (MP65) and heat shock protein 90 is mediated by antibody fragments (Fabs) and does not require Fc regions (143, 147). Notably,

antibodies to *C. albicans* MP65 and secretory aspartyl proteinase-2 that lack Fc regions were shown to inhibit fungal adherence to epithelial cells (149).

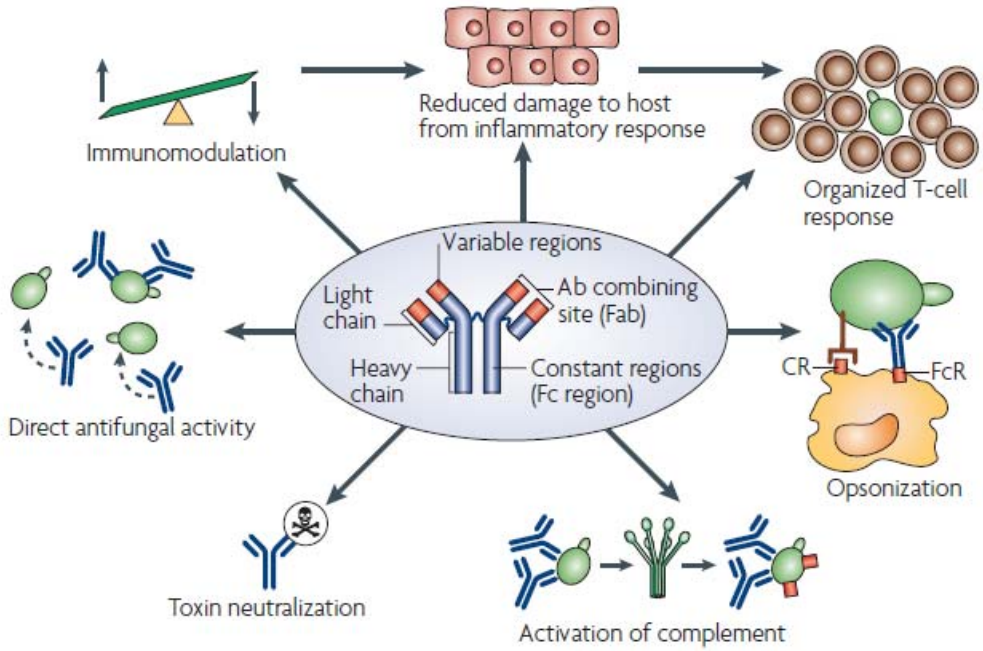


Figure I: Antibody-mediated protection against fungal diseases (Cutler *et al* 140)

The efficacy of antibody fragments against experimental candidiasis in mice suggests that these fragments may hold promise for avoiding the formation of potentially detrimental immune complexes and unwanted antibody responses to Fc regions. However, since Fc regions are necessary for the efficacy of antibodies to other fungal targets, a greater understanding of mechanisms of antibody efficacy against different fungal targets is needed to develop rationally based therapeutic antibody agents for fungi. While there is currently insufficient evidence to suggest that antibodies to fungal targets are more effective against systemic disease or mucosal disease, or both, it is possible that antibodies that mediate protection by blocking adherence might be more effective against mucosal disease, whereas antibodies that mediate protection by enhancement of effector cell phagocytosis might be more effective against systemic disease.

The mechanisms of antibody-mediated defence against fungi include direct antibody (Ab) neutralization of fungal toxins and extracellular enzymes, and

direct inhibition of fungal growth (150). Antibodies can indirectly inhibit fungi by functioning as an opsonin, either alone or in conjunction with complement factor C3, which is activated and deposited as C3b and which degrades to iC3b on the fungal surface (151, 152). Antibody and complement-coated fungal cells interact with Fc receptors (FcR) and complement receptors (CR) on host phagocytic cell membranes, resulting in prompt ingestion of the fungal cell and which can lead to the death of the ingested fungal cell (153). In defence against intracellular fungal pathogens, such as *Cryptococcus neoformans*, protective antibodies seem to have a role in modulating host inflammatory responses and enhancing the organization of T-cell responses (47, 148, 154).

The criteria for the possibility of protective antibodies against candidiasis and other fungal diseases are as follows:

1. Specificity. The experience with *C. neoformans* and *C. albicans* indicates that specificity is a critical variable in determining antibody efficacy. *C. neoformans* protective antibodies against cryptococcosis are specific for glucuronoxylomannan (155, 156). However, not all antibodies to glucuronoxylomannan (GXM) are protective suggesting that individual epitopes in this polysaccharide antigen differ in their ability to elicit protective and non-protective MAbs (157). For *C. albicans* antibodies against anti-b-1, 2-mannotriose or anti heat shock protein (HSP90) are effective (158, 159).

2. Ig isotype. The experience with *C. neoformans* suggests that antibody isotype is an important variable in determining the efficacy of antibodies against fungi. However, the relative efficacy of the various isotypes may differ depending on the fungal pathogen.

3. Titer. Antibody-mediated protection against fungal pathogens is likely to depend on an optimal amount of serum antibody. For *C. neoformans*, passive antibody has been found to be effective only at certain doses (140).

2.3.2.2.1. Mannan and Stress Mannoprotein-specific Monoclonal Antibodies

Mannan is found in the cell wall as large N-linked and shorter O-linked manno oligosaccharides associated with mannoproteins.Anti-mannan antibodies are prevalent in human sera, including patients and normal population (141, 142). The mannan component is also involved in adhesive interactions (125). In one of the reports, immunization of mice with a mannan fraction (encapsulated into liposomes) induced protective antibody responses against *C. albicans*. Two monoclonal antibodies specific for different mannan epitopes in the adhesin fraction were found and both antibodies agglutinated *Candida* cells but only one of them protected mice against disseminated candidiasis. The protective antibody recognized the acid-labile adhesins while the non-protective antibody recognized a different epitope in the fraction (143, 144). The ability of the antibody to rapidly deposit high amounts of complement factor C3 onto the yeast cell wall is needed for protection against disseminated candidiasis (145). Anti-mannan antibodies can also mediate protection in animal models of *Candida* vaginitis (146, 147). Combined detection of mannanemia and anti-mannan antibodies may have diagnostic value and also help monitor patients with candidiasis (148).

2.3.2.2.2. β-D-Glucan-specific Antibodies

The protective role of β-D-Glucan-specific antibodies was confirmed by passive immunization of naïve mice with sera or purified IgG obtained from vaccinated mice. Anti-BG antibodies (Abs) might enhance *in vitro* candidacidal activity of human macrophages, suggesting their ability to influence the host defense against *Candida* through opsonisation. Interestingly, patients with deep mycosis have a significantly lower titer of anti-BG Abs compared with normal individuals. Furthermore, anti-BG Abs titer decreases remarkably in mice, following intravenous administration of BGs. This suggests the formation of a complex between anti-BG Abs and *Candida* cell wall BGs, which could promote the clearance of the pathogen from the systemic circulation. A decreasing of the anti-BG Abs titer in mycoses patients can be reverse correlated to the progression of fungal infection. These and other observations have supported a protective role of these antibodies (137,166).

2.3.2.2.3. SAP-specific Vaginal Fluid Antibodies

Protection of SAP-specific antibodies was confirmed by the passive transfer experiments in which vaginal fluid from previously infected rats was applied intra vaginally to naïve rats which were thereafter protected against experimental VVC. When the vaginal fluids were adsorbed on SAP particles, before being transferred to recipients, their protective effect was diminished or abolished (164).

2.3.2.2.4. Hsp90-specific Monoclonal Antibodies

From a broad spectrum of human serum antibodies specific to serum 47 kDa breakdown fragment of *Candida* Hsp90 only a few epitope-specific antibodies were characterized thoroughly. They recognize epitopes localized in ATPase domains of Hsp90 (a) the EEVD motif localized on the C terminus, (b) the QQNKILKVI motif involved in binding of the ATP molecule, (c) the partially overlapping LKVIRK motif which is necessary for *Candida* growth and (d) the LSREM motif recognizing γ-phosphate in the ATP molecule which is subsequently accessible to reaction with nucleophilic water. Binding of antibodies to these domains inhibits the Hsp90 functions in cell-wall synthesis. Intensive biotechnological research in the field of QQNKILKVI-specific antibody allowed the generation of histidine-conjugated Ig fragment which recognizes the peptide region surrounding amino acid 330 where QQNKILKVI is located. This recombinant antibody-based protein (Mycograb®; Neu*Tec Pharma*, USA) was expressed in *E. coli* under standard laboratory conditions (167, 168). Experimentally and clinically Mycograb® is able to sustain the inhibitory effect of liposomal drug formulation of amphotericin B or echinocandin in human disseminated candidiasis caused by *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. lusitaniae* (169). Mycograb® is being tested in a multicentre clinical efficacy trial III (169). The Hsp90 domains involved in molecular chaperone activity are still not properly identified and might not be necessarily localized within the 47-kDa fragment. The generation of antibodies that recognize such domain requires immunization of experimental animals with the N´-terminal part of Hsp90 (or entire Hsp90). Immunization with

various *Candida* Hsp90 fragments can elicit antibodies with intense anti-*Candida* activity even stronger than Mycograb®. Theoretically, new approaches to the treatment of candidiasis are now available (170). Indeed, intradermal (i.d.) immunization of mice with full-length recombinant *Candida* Hsp90 protein induced a protective response which improved the survival of subsequently i.v. infected mice (169). Because the recombinant Hsp90 was expressed in *E. coli* it was reasonable to expect that a more appropriate post-translational modification would be achievable after *Hsp90* expression in a eukaryotic system. For this purpose, mice were immunized intramuscularly with *Candida* Hsp90-coding DNA plasmid. After H*sp90* DNA vaccination similar, but less impressive, results were obtained (168). The decrease in protection was probably caused by the reduced amount of antigen and by the shift in cytokine responses after intramuscular DNA vaccination compared to i.d. administration of recombinant Hsp90. The protection conferred by *Candida* Hsp90-specific antibodies was experimentally confirmed only in systemic candidiasis.

2.3.2.2.5. Involvement of *Candida*-specific Antibodies in Human VVC

The contribution of various vaginal *Candida*-specific antibodies to protection of women against VVC is still controversial. Women suffering from VVC have relatively high serum and vaginal *Candida* specific antibodies of IgG4, IgG1, and IgA isotypes and the increase in antibody titers directly correlates with the increase in *Candida* load (171, 172). Little *et al.* (2000) reported that RVVC-suffering women responded to *Candida* infection by increasing the serum level of T-cell-derived antigen binding molecules (TABMs) associated with TGF-β and IL-10, cytokine characteristic for regulatory T-cell subsets. TABMs are presented in serum during systemic T_H2 type humoral immune response and deliver associated cytokines to the antigen-specific T cells to suppress their inflammatory response. Although this resembles the protective role of *Candida*–specific Treg responses, clinically those women still suffer from inflammatory RVVC. Whether these Treg and cytokines do not offer sufficient immune suppression to counter-balance the inflammation generated by PMNs

or whether other factors play a role in VVC and RVVC remains to be elucidated further (145, 146).

2.3.2.2.6. Involvement of *Candida*-specific Antibodies in OPC

The involvement of *Candida*-specific antibodies in protection against OPC is not clear. During human OPC, the titers of specific antibodies increase proportionally to *Candida* load, similarly to human VVC. Human saliva *Candida*-specific IgA isotypes inhibit adherence of *Candida* to buccal epithelial cells whereas other isotypes do not have this activity. Inhibition of the *Candida* adherence by IgA could be abrogated by other isotypes and/or by antibodies specific to *Candida* antigens that are not involved in adherence. This could be the explanation for many other studies that did not confirm any protection (147). Many cytokines are involved in the activation of mucosal Ig-producing plasma cells. Pro inflammatory (T_H1 type) cytokines IL-12 and IFN-γ induce isotype switching to high-quality opsonising antibodies (148). Nevertheless, in mouse experimental OPC, T_H2 cytokine IL-4 was required for effective clearance of *Candida* (149). Isotype switch to most promising IgA isotype is considered to be induced by anti-inflammatory cytokine TGF-β. Subsequent translocation of polymeric IgA through epithelial cells is induced by IFN-γ, TNF, and IL-4. Thus both T_H1 and T_H2 cytokines could be involved in protective antibody response during OPC and their individual proportion is probably influenced by fungal burden, time frame of OPC, and by the OPC-causing immune imbalance (173).

2.4. Antifungal Resistance: Epidemiology and Mechanism

Several classes of antifungal drugs are used to treat fungal infections in humans. Depending on their antifungal properties, these drugs are used in specific clinical situations and for specific fungal species (30). The use of these drugs is further restricted by their safety, development of resistance by the fungus and their limited effectiveness profiles. Antifungal resistance can be defined as phenomenon describing failure of a fungal infection to respond to antifungal therapy. Antifungal resistance has been traditionally classified as primary, secondary and clinical resistance. Primary Resistance is the intrinsic

property of fungi present before exposure to antifungal agents. Secondary or Acquired resistance is the resistance that develops after exposure to antifungal agent owing to stable or transient genotypic alterations. Clinical resistance is the progression or relapse of an infection by a fungal isolate that seems, in laboratory testing, to be fully susceptible to the antifungal used for the treatment of infection. Clinical resistance of fungi is typically seen in patients with persistent, profound immune defects (e.g. AIDS, neutropenia), or infected prosthetic material such as central venous catheters.

Understanding the mechanism of action of different antimicrobial agents is an important prerequisite to understanding mechanisms of resistance. The emergence of drug resistance is an evolutionary process that is based on selection for organisms that have an enhanced ability to survive and reproduce in the presence of a drug. In competitive and communicative microbial communities in nature, microorganisms invest considerable energy in the production and elaboration of antimicrobial agents (182). Consequently the evolution of drug resistance is ubiquitous in nature, and microorganisms explore diverse strategies to out combat the drug. The fact that the emergence of drug resistance out paces the development of new antimicrobial agents underscores the crucial importance of understanding the evolutionary mechanisms that lead to the development of resistance.

The discovery of new antifungal agents should be based on consequences of better knowledge of molecular mechanism of pathogenicity, virulence factors and their contributions in infections as well as on the recent developments in genomic and proteomic technologies. Besides, as fungal infection results from failure of the host immune system to eliminate pathogens, immunotherapy designed to enhance host defence mechanisms against candidiasis may be very useful in combined treatments. This approach requires further studies allowing a better understanding of the mechanisms used by the host immune system against fungal pathogens. The list of currently available antifungal agents has been listed in Table I.

2.4.1. Polyenes

Polyenes constitute as class of natural antifungal compounds discovered 50 years ago. Amphotericin B (AMB) and nystatin are the two clinically useful members of this group. For many years, AMB was the only therapeutic agent of value to treat invasive fungal infections. Because of its broad spectrum of activity, it earned the title of “gold standard” in antifungal therapy. The target of amphotericin B is the fungal membrane, on which they act directly. Amphotericin B binds to ergosterol leading to the formation of aqueous pores consisting of an annulus of eight amphotericin B molecules linked hydrophobically to the membrane ergosterol.

Intrinsic or primary resistance to amphotericin B has been reported along with the increase in incidences of invasive infections due to more number of emerging yeast like fungi such as *Trichosporon beigelii, C. lusitaniae* or *C. guillermondii*, and filamentous fungi like *Fusarium* species, *Scopulariopsis* species, *Scedosporium* species, and some dematiaceous fungi (183, 184). Secondary or acquired resistance to amphotericin B has limited occurrence till now. However, isolated cases of acquired resistance to amphotericin B have been described, especially in yeasts causing infections in patients with cancer (185). Furthermore, yeast isolates from patients undergoing myelosuppressive chemotherapy or bone marrow transplantation were seen to have significantly higher MICs to amphotericin B than colonising isolates from immunocompetent patients (186).

Strains of *C. albicans* acquiring resistance to amphotericin B or amphotericin B in combination with azoles have been reported in patients receiving treatment with these antifungals (187, 188, 189). Acquisition of polyene-resistant species such as *C. lusitaniae* and *C. guillermondii* has also been described during amphotericin B therapy (186, 188). The development of secondary resistance to amphotericin B is alarming, particularly among immunocompromised patients that may require long-term antifungal prophylaxis and/or treatment. The fungicidal activity of amphotericin B is

widely believed to occur once drug binds to ergosterol in the fungal cell membrane and induces the formation of pores in the phospholipid bilayer. This

Table I: Antifungal Agents, their spectrum and mode of action

Antifungal	Mechanisms and action	Spectrum/Comments
Polyenes Amphotericin B Nystatin	Interaction with ergosterol and destabilization of cell memberane functiom;cell death	Broad sensitivity against *Candida* spp. *Cryptococus neoformeans* fungi except of the *Aspergilius* spp, *A.terreus* and *A.flavus*.
Pyrimidine analogue 5-flurocytosine	Interferes with DNA/RNA synthesis	Activity against *Candida* spp. *Cryptococus* spp.Rapid emergence of resistance used as momotheraphy
Azoles Ketoconazole Fluconazole Itraconazole Voriconazole Posaconazole	Inhibition of Cytochrome P450 14α-lanoserol demethylase	Fluconazole isa active against *Candida* spp. *Cryptococus* spp but has no activity against invasive molds. Other azoles such as Itraconazole**,**Voriconazole and Posaconazole have improved activity against invasive molds..
Echinocandins Caspofungin	Inhibition of cell wall glucan synthesis, leading to susceptibility of fungal cell to osmotic lysis	Rapidly fungicidal against *Candida* spp and moderate against *Aspergilius* spp,including azole resistant species. Poor activity against *Cryptococus neoformeans*
Allylamine Terbinafine	Inhibition of squalene epoxidase	Activity aginst most dermatophytes ,poor activity against *Candida* spp

action results in altered membrane permeability and cell death (189). The exact mechanism of resistance of amphotericin B has not been clearly understood. While several mechanisms have been proposed, the best proposed mechanism of resistance to amphotericin B may involve the cell membrane possessing altered sterol composition. These mutations may lead to the production of unusual sterol types, alter the stereochemistry of membrane ergosterol, and/or decrease the overall quantity of ergosterol in the cell membrane may result in diminished amphotericin B binding capacity and thus lead to decreased efficacy (190, 191).

Polyene resistance has been best defined among *Candida* species, and is commonly attributed to a defect in the ergosterol biosynthetic pathway. Nolte

and colleagues (1997) reported resistance among *C. albicans* to fluconazole and amphotericin B isolated from two patients with leukaemia may be due to altered activity of $\Delta^{5,6}$-sterol desaturase. This mutation led to the increased production of abnormal membrane sterols, including 3β-ergosta-7, 22-dienol, and a decreased production of ergosterol which was also reported in a polyene-resistant strain of *C. albicans* isolated from a patient with AIDS (192, 193). Inactivation of the $\Delta^{5,6}$-sterol desaturase enzyme occurs secondary to a mutation of the *ERG3* gene may also affect azole antifungal activity (194). Another alteration in *ERG11* gene in ergosterol biosynethic pathway also results in polyene resistance among *C. albicans* (195). Sanglard and colleagues (2003) reported the development of a mutant with defects in the *ERG11* gene in the presence of amphotericin B *in vitro* (196).

Among other *Candida* spp., mechanisms of resistance correlate with a defect in ergosterol synthesis too. A mutation in the *ERG6* gene among *C. glabrata* leading to decreased membrane ergosterol and increased sterol intermediates has been associated with reduced susceptibility to the polyenes (197, 198). An analysis of *C. krusei*, *C. parakrusei*, and *C. tropicalis* revealed that resistant strains had a decrease in the quantity of membrane sterols when compared with wild-type strains (199). In addition to alterations in ergosterol synthesis, oxidative changes within the fungal cell may also play a role in polyene resistance among *Candida* species. Following exposure to erythromycin, isolates of *C. albicans* demonstrated decreased susceptibility to amphotericin B, which likely occurred secondary to a decrease in aerobic respiration (200). Because ergosterol synthesis is oxygen-dependant, alterations in cellular oxidative processes may lead to decreased ergosterol production and subsequent resistance to amphotericin B (201). Further evaluation of the role of oxidative processes in polyene resistance is required in order to better characterize the implications of these changes among *Candida* species.

2.4.2. Azoles

Azoles are the most widely used antifungal agents. Azoles were first introduced in the 1960's as N-substituted imidazole derivatives such as

miconazole, econazole, and clotrimazole. These azoles were followed by the introduction of first generation triazoles such as fluconazole, ketoconazole and itraconazole. Both fluconazole and itraconazole displayed a broader spectrum of antifungal activity than the imidazoles and had a markedly improved safety profile compared with amphotericin B and ketoconazole. Despite widespread use, they exhibit a number of clinically important limitations such as suboptimal spectrum of activity, the development of resistance, the induction of hazardous drug–drug interactions, and toxicity. In order to overcome these limitations, several analogues have been developed. Second generation triazoles such as posaconazole, voriconazole and ravuconazole have greater potency and possess increased activity against resistant and emerging pathogens (202).

The mode of action of azoles is based on the inhibition of ergosterol biosynthesis at different step (202, 203). Cytochrome P450 lanosterol 14α-demethylase, encoded by the gene *ERG11* gene is the point of action by many azoles. The 14-methyl group of lanosterol is important to generate 14-methylated intermediates; one of these intermediates, 14-methylergosta-8, 24(28)-dien-3, 6-diol, is toxic and is responsible for the fungal inhibition (204). Increase in azole resistance in *C. albicans*, especially fluconazole resistance among HIV-infected patients with oropharyngeal or oesophageal candidiasis is a direct result of increase in the use of itraconazole and fluconazole (192). Although azole-resistance is less common among the patients with vaginal candidiasis and candidaemia, about one third of patients with advanced AIDS harbour fluconazole resistant *C. albicans* in their oral cavities (193).

Candida species employs four major mechanisms against azole compounds.

1. Decreased drug concentration: the development of efflux pumps results in the decreased drug concentrations at the site of action. Two gene families' viz., *CDR* genes of the ATP-binding cassette super family and the *MDR* (Multi-Drug Resistance) genes of the major facilitators' class. *CDR* gene up-regulation confers resistance to almost all azoles, while *MDR*-encoded efflux pumps have a narrower spectrum specific for fluconazole (205).

2. Target site alteration: Mutations in the target enzyme gene, *ERG 11*, which encodes lanosterol 14α-demethylase prevents binding of azoles to the enzymatic site. Intrinsic resistance of *C. krusei* to fluconazole has been attributed to the decreased affinity of lanosterol 14α-demethylase to the azole. Different mutations can coexist in the same gene with additive effects (195).

3. Up-regulation of target enzyme: Some *Candida* isolates with reduced susceptibility to azoles have higher intra-cellular concentrations of *ERG11* protein (31). This can be achieved through gene amplification, increased transcription or decreased degradation of the gene product. This mechanism is thought to contribute very little to the overall resistance.

4. Development of bypass pathways: Exposure to azole compounds results in depletion of ergosterol from the fungal membrane and accumulation of the toxic product 14α-methyl 3, 6-diol, leading to growth arrest. Mutation of the *ERG3* gene prevents the formation of 14α-methyl 3, 6-diol from 14α-methyl fecosterol leading to the accumulation of other sterols instead of ergosterol (196). Alternative genes are expressed which bypass the pathway.

2.4.3. 5-Flucytosine

5-Flucytosine (5-FC) is a base pyrimidine analog that inhibits cellular DNA and RNA synthesis. It is effective against many types of yeast including *C. albicans* and *Cryptococcus neoformans.* 5-FC enters the fungal cell with the help of cytosine permease, and deaminated into 5-fluorouracil by cytosine deaminase. 5-flucytosine is apparently converted by cellular pyrimidine-processing enzymes into 5-fluoro-dUMP (FdUMP). FdUMP is a specific inhibitor of thymidylate synthetase, an essential enzyme for DNA synthesis, 5-fluoro-UTP (FUTP) is incorporated into RNA, thus disrupting protein synthesis. 5-FC is a fungus specific drug, since mammalian cells have little or no cytosine deaminase (206).

2.4.4. Allylamines

Allylamines, such as terbinafine and naftifine, have been developed as a new class of ergosterol biosynthetic inhibitors that are functionally as well as

chemically distinct from the other major classes of ergosterol-inhibiting antifungal agents (211). Allylamines act by inhibiting early steps of ergosterol biosynthesis. This inhibition coincides with accumulation of the sterol precursor squalene and the absence of any other sterol intermediate (212). Although clinical failure has been observed in patients treated with terbinafine, allylamine resistance in association with clinical use of terbinafine and naftifine has not been found in human pathogenic fungi. Mechanism of resistance to allylamine is poorly understood.

2.4.5. Echinocandins

Echinocandins exhibit fungicidal activity both *in vitro* and *in vivo* against *Candida* and *Aspergillus* species by inhibiting the synthesis of β-1,3-D glucan, which is an integral part of the cell wall. Echinocandins are specific non-competitive inhibitors of β-1,3-D-glucan synthase, a large integral membrane heterodimeric protein (212). They also have secondary effects on other components of intact cells including a reduction in the ergosterol and lanosterol content and an increase in the chitin content of the cell wall. Inhibition of β-1,3-D-glucan synthetase results in cytological and ultra structural changes in fungi characterized by growth as pseudohyphae, thickened cell wall, and buds failing to separate from mother cells. Cells also become osmotically sensitive (213). Echinocandins are ineffective against Zygomycetes, *Cryptococcus, Trichosporon,* and *Fusarium* species. Among *Candida* species *C. parapsilosis* show increased MIC values (214).

The mechanisms of resistance to echinocandins are still being investigated. In *Candida* species, secondary resistance is associated with point mutations in the *Fks*1 gene of the β-1,3-D-glucan synthase complex (208). The mechanism of resistance in *C. neoformans* is not completely understood. Possibilities include echinocandin-resistant β-1,3-D-glucan synthase target, efflux pumps, and degradation pathways (209).

2.5. Diagnosis of *Candida albicans* Infections

The first essential component of antifungal treatment is the correct and prompt diagnosis as this is the most important factor associated with improvement of

the management of fungal infections in immunocompromised patients. The management of opportunistic fungal infections is characterized by a series of unresolved problems including initial difficulties in obtaining an early diagnosis. Most superficial and subcutaneous fungal infections are easily diagnosed but diagnosis of systemic fungal infections is difficult and many infections are confirmed only after autopsy (215). The clinical symptoms of systemic candidal infections are nonspecific and similar to those of bacterial and viral infections. In addition, the isolation of fungi from clinical samples is unreliable and may be complicated by the presence of a colonising commensal organism, or ubiquitous fungi in the environment, causing false-positive results (216). Also, the collection of clinical samples often requires an invasive procedure, which may not be advisable in critically ill patients. Precise diagnosis of the causative agent is required in order to start an appropriate therapy that will not only help in controlling the causative organism but also avoid the side effects associated with wrong therapy and the development of drug resistance by the organism. A multidisciplinary approach is required to both diagnosis and disease management.

A diagnostic method should be specific for a particular species or a genus or a group of similar pathogenic organisms, must be rapid, and should be easy to assess without any ambiguity in drawing the conclusions. Various methods are available for the diagnosis of *Candida* infections. Conventional methods such as microscopic examination of clinical samples may be a direct method to identify the casual organism but this method is often ambiguous and difficult to confirm due to the presence of sparse fungal populations. Histopathology and biopsy of the infected tissue is invasive and cannot be followed in most cases. Culturing of the pathogen from the blood, joint fluid, cerebrospinal fluid, vaginal lavage, brocheoalveolar lavage, oesophageal brushing, sputum, urine and stool is also a direct method but lacks sensitivity, specificity and often leads to false positive results as *C. albicans* is a commensal and is also present in the normal population and some of these techniques are invasive and not usually recommended for diagnosis. During the last two decades, much effort has been made to develop reliable tests for rapid diagnosis of

invasive candidiasis leading to appropriate therapy. Most of them are indirect but are less invasive, sensitive, rapid and cost effective. However, each test has its own advantages and disadvantages (6).

PCR based detection technique has gained importance in the recent times. A range of PCR targets have been used for this purpose including cytochrome P450 genes, heat shock protein genes and genes involved in pH regulation, r RNA genes. Ribosomal RNA genes are most frequently used because of their universal nature and presence in large copy numbers. Also a variety of post amplification techniques have been employed to improve the sensitivity and specificity of the PCR based technique. These include nested PCR, restriction fragment length polymorphism (RFLP), PCR–enzyme-linked immunosorbent assay (ELISA), single-strand confirmation polymorphism, hybridization with specific probes and sequencing. These techniques are rapid and sensitive (87). The PCR based techniques have their own disadvantages such as low abundance of the fungal DNA in the patient samples for amplification as it is degraded very quickly, the need for designing specific primers which shows no cross reactivity with the other species. Also any contamination of the sample with the DNA from other organisms, sharing the same evolutionary niche, may lead to false positive results. Recent advances in PCR based diagnostic technique includes quantitative PCR (qPCR) or real- time PCR which utilizes fluorescently labelled specific probes. The technique is very sensitive and samples with very low levels of DNA can also be utilized. This technique also removes the need for post-amplification handling, reducing both turnaround time and the potential for contamination, and provides a species/genus level of identification, depending on the design of the probe (212). However the sensitivity also increases the chances of false positive results in case of contamination. The technique is also costly and not available commercially. Advanced techniques such as CT scanning, radiographic methods have been developed more recently but they are costly and not available commercially (213, 214).

Serological tests that detect antibodies against various metabolites of the pathogen such as β-1,3-D-glucan, D-arabinitol, mannans, various glycoproteins and proteins such as enolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate 3-kinase, heat shock protein (HSP90) etc have been employed as a diagnostic criteria for the identification of the fungal pathogens. These antigens are highly immunogenic and elicit a strong humoral response resulting in the production of antibodies by the patient's immune system which can be detected *in vitro* by using techniques such as agar gel diffusion, latex agglutination, ELISA, chemiluminiscence ELISA. However serological detection is low in sensitivity and specificity because many patients with systemic fungal infections are immuno-compromised and therefore have an impaired antibody response. Even in immuno-competent individuals, the delay between the onset of infection and the development of the antibody response reduces the practical value of these tests.

2.6. Covalently Linked Proteins of *Candida albicans* Cell Wall

The cell wall is an essential structure that maintains the viability of fungal cells, conferring their typical morphology and protecting the cell against external injuries. The Cell wall is the structure that: (i) first comes into contact with host cells; (ii) carries important antigenic determinants of the fungus; (iii) is responsible for the adherence of the pathogen; and (iv) establishes a cross-talk with the host, which depends on what has been referred to as the 'glycan code', which includes modifications in the chemical composition and linkages of the cell wall polysaccharides. As the most external cellular structure of pathogenic microorganisms, it also carries important antigenic determinants and mediates adhesion to the host tissues, being therefore crucial to initiate colonization and therefore, cause disease (49).

Candida albicans uses an arsenal of molecular tools to overcome the host lines of defense. An important role in the fitness and virulence of *C. albicans* is reserved for those cell wall proteins (CWPs) that are covalently linked to the skeletal cell wall polysaccharides. They contribute to cell wall integrity, mask

the β-glucan layer, thus avoiding detection by dectin-1, promote biofilm formation, mediate adherence to host cells and abiotic medical devices, promote invasion of epithelial layers, offer protection against the attacks of the innate immune system, and are involved in iron acquisition. CWPs of C. albicans have been extensively studies in recent years, reflecting the growing realization of how important their role is in the various stages of infection (215, 216, 217, 218). Gel free proteomics of isolated walls has shown that at any time covalently linked CWPs profiles can change dramatically depending on the environmental conditions. In addition, the presence of particular CWPs seems to be niche specific and strongly correlated with either yeast or hyphal growth of *C. albicans*.

The covalently linked CWPs in the protein coat of the wall are the rank and file in the ongoing battle between *C. albicans* and its host. They also play a major role in the development of biofilms and in the interactions with other microbial organisms. Nevertheless, detail knowledge about their precise function is in many cases still incomplete (219). Their precise location and their 'visibility' to the immune system are also often unknown. The CWPs which possess a carbohydrate-binding module and might therefore be involved in cell wall construction, in the synthesis of the extracellular matrix of biofilms, or even in the recognition of host ligands is has not been fully characterized yet (220). The protein composition of the wall can vary widely during infection, this argues for determining the changes in CWP profiles under infection-associated conditions. Such responses are still relatively unexplored.

The cell wall fractionation reveals that the major CWPs can be divided into two classes: (i) those cell wall proteins which can be extracted by hot water, but mainly by ionic detergents or chaotropic agents (NCL-CWP) and (ii) secondly those proteins which are resistant to this treatment, and solubilised only after digestion of the structural polysaccharides, or by breakage of specific bonds through which they are bound to wall polysaccharides (49). This second group of proteins has been divided into two subclasses. One subclass of protein include glycoproteins covalently linked to the cell wall which are referred as

'true' CWPs. Proteins from another subclass are devoid of a carbohydrate moiety and are retained in the wall by unknown mechanisms. There are three different groups of covalently bound glycoproteins. Group I includes proteins bound to β-1, 6-glucans through a GPI moiety (GPI CWP) (24, 221). Group II corresponds to Pir proteins (proteins with internal repeats), characterized as containing repetitive sequences and being highly O-glycosylated (222). Pir proteins are attached to β-1,3-glucan by unknown alkali-sensitive bonds (possibly O-glycosidic linkages) (ASL-CWP). Recently, some proteins without internal repeats but bound through alkali-sensitive bonds have been reported (Figure II) (24, 223).

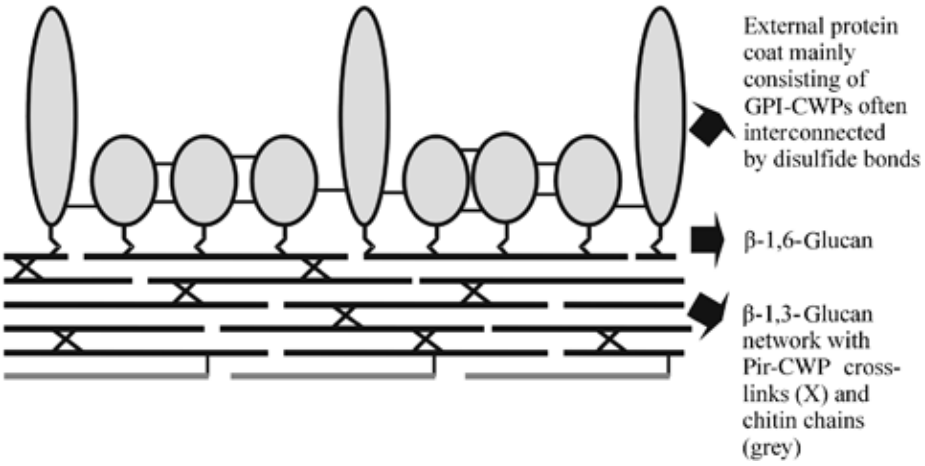


Figure II. The cell wall organisation in *Candida albicans* (Klis *et al*, 2009)

2.6.1. *Candida albicans* GPI CWPs

GPI cell wall protein shares major fraction of covalently linked cell wall proteins of *C. albicans*. These are covalently linked proteins linked with CWPs through a GPI-remnant to β-1,6-glucan (GPI CWPs). The β-1,6-glucan moiety may in turn be linked to β-1,3-glucan or to chitin. GPI proteins possess two signal peptides located at either end of the polypeptide chain. The N-terminal signal peptide directs them to the endoplasmic reticulum (ER). In the ER, the N-terminal signal is removed and the C-terminal signal is replaced by a preassembled lipid anchor known as GPI anchor that links the proteins to the luminal leaflet of the membrane. Fungal GPI proteins follow the secretory

pathway until they reach the plasma membrane, where some of them are retained (GPI plasma membrane proteins such as Ecm331), whereas others are released from the plasma membrane and incorporated into the wall (224, 225). Genome wide *in silico* analyses identified 115 putative GPI proteins in the genome *of C. albicans* (226, 227). These GPI proteins can either be bound to the plasma membrane or are incorporated into the cell wall, depending on the carboxy-terminal protein sequence (228, 229). GPI cell wall proteins displaying various functions as some GPI proteins are important for physical strength, permeability of the cell wall or adhesion properties, whereas others have been shown to exhibit enzymatic functions. As GPI CWPs are linked to the skeletal polysaccharides through their C-terminal end, their N-terminal part is facing outwards. The presence of a particular GPI protein in the cell wall does not exclude the possibility that a considerable number of copies or even the majority are retained in the plasma membrane, and vice versa (24).

2.6.2. Mild Alkali Cell Wall Proteins

It contains the CWPs covalently linked to β-1,3-glucan directly via an alkali labile linkage an O-glycosydic linkage. One of the known proteins in this family is Pir (Protein with Internal Repeats). Their presence in ascomycetes and deuteromycetes appears to be universal, and their organization is similar, including the presence of a signal peptide, a Kex2 sensitive site, a domain with two to 11 repetitive sequences, and a C-terminal sequence with four Cys residues at identical positions. Pir proteins do not contain a GPI anchor motif and are attached to the cell wall by unknown alkali-labile bonds, possibly O-glycosidic linkages with β-1,3-glucan. Some of the Pir proteins are retained in the wall exclusively by disulfide bridges, given that some of them are released by reducing agents such as β-ME or DTT (228, 229). In *C. albicans*, an antibody directed to the *S. cerevisiae* Pir protein Hsp150 recognized two proteins extracted by alkali or β-1,3-glucanase, and a high-molecular-mass protein secreted to the growth medium, demonstrating the existence of Pir related proteins in the fungus (230, 231). In similar experiments, Western blot analysis using an antiserum directed against *S. cerevisiae* Pir2p/Hsp150

revealed the presence of at least two differentially expressed Pir2 homologs in the cell surface of *C. albicans* (232). It was also observed that in Dmnn9 and Dpmt1 mutants, which are defective in N- or O-glycosylation, respectively, as well as in a Dkre6 mutant, the amounts of Pir proteins were slightly up-regulated (233). Recent results have revealed that C. *albicans* contains a single Pir-protein encoding gene. By use of mass spectrometry and *in silico* analyses, two Pir proteins encoded by non identical alleles of a single gene (CaPIR1) were identified (234, 235). Both encoded proteins contained a single N-mannosylated chain, four Cys residues and seven repeats, but one of them was 21 amino acids shorter. Homozygous mutants were impossible to obtain, suggesting that the gene is essential for growth. Heterozygous mutants displayed an abnormal phenotype associated with wall alterations. In this regard, in a parallel study, similar analysis of NaOH-released proteins led to the identification of two Pir proteins Pir1 and Pga29 (228).

2.6.3. Functions of Covalently Linked Cell Wall Proteins

1. Coat-forming CWPs:

Pga59 is a likely candidate for a coat-forming CWP that restricts the permeability of the cell wall (236).

2. Hydrophobicity-conferring CWPs:

Eap1 promotes adhesion to styrene, a hydrophobic polymer derived from ethenylbenzene (237).

3. The ALS adhesin family:

It consists of eight GPI proteins, seven of which have been experimentally validated as covalently linked CWPs. The mature Als proteins, which have lost their N- and C-terminal signal peptide, are multi domain proteins with four consecutive domains (Ig–T–TR–stem; Ig, immunoglobulin; T, threonine-rich domain of Als proteins; TR, tandem repeat domain of Als proteins): the Ig domain of C terminal 300 amino acids, a region with three tandem Ig-like sequences; the T-domain, a 127-residue threonine-rich conserved domain; the TR domain, a region consisting of a variable number of 36-residue, threonine-

rich, tandem repeats; and the stem domain, a highly glycosylated Ser/Thr-rich domain of low structural complexity and variable length (238, 239). Although it was originally thought that the Ig domain was solely responsible for the adhesive properties of the ALS proteins and the remainder of the protein was functioning as a stalk domain, it is now clear that the T-domain and the TR-domain have vital functions of their own, particularly in cell–cell aggregation. In view of the multiple functions assigned to ALS proteins, it therefore becomes important to establish which domain is directly responsible in each case. ALS proteins bind to diverse mammalian proteins. One of the reasons is that the recognition of peptide ligands by the ALS proteins is degenerate and that their specificities overlap each other only partially. This allows *C. albicans* to bind to a large variety of host proteins (240). Importantly, some members of the ALS adhesin family have amyloid properties (238, 241, 242). This property probably contributes to intercellular aggregation and biofilm cohesiveness (243, 244). ALS proteins are also involved in the formation of mixed aggregates consisting of bacterial and fungal cells.

4. The adhesin Hwp1:

It represents a fascinating case of molecular mimicry. Its N-terminal domain is recognized as a substrate by host transglutaminases at the epithelial surface. As a result, *C. albicans* becomes covalently linked to epithelial cells and cannot be washed away (245). Hwp1 also plays a complementary role in biofilm formation, together with Als1 and 3, possibly as a result of a physical interaction between Als1 and 3 on the one hand and Hwp1 on the other (246).

5. Pir family:

It is a cross-linking CWP. Consistent with its location in the internal skeletal layer, Pir1 presumably cross-links b-1,3-glucan chains (234, 247).

6. Carbohydrate-active enzymes:

Many CWPs has been predicted for having glycosylase/transglycosylase activity (228, 248). Their location in cell wall is not certain. These proteins are may be part of the external protein coat and thus located far away from their

presumptive substrates or might be located elsewhere in the wall. Alternatively, they could play a role in the formation of the extracellular matrix of biofilms. Loss of the putative cell wall (trans) glycosidase Sun41p, a non-GPI CWP, results in strongly decreased biofilm formation on an abiotic surface (249, 250).

7. Coping with oxidative stress:

C. albicans incorporates the GPI modified superoxide dismutases Sod4 and 5 into its wall, which help the cell to cope with oxidative stress originating from innate immune cells (238, 251, 252, 253, 254).

8. Invasion-related CWPs:

Other CWPs, such as Als3, have been shown to act as an invasin, thereby facilitating endocytosis (255).

9. The yapsin-like proteins:

Sap9 and 10 possess proteolytic activity, and in their absence, normal cell wall construction is affected, but their specific substrates are still largely elusive (256, 257).

2.7. Role of Proteomics, Bioinformatics Tools in Target Identification

Proteome is the total number of proteins present in a cell at a given time under given condition and its study is called as proteomics. Proteomics offers a global and integrated view of the entire protein complement expressed by the genome. Several thousand proteins are expressed under a given condition at a time. Some of them may be very important for the survival of the pathogen under the given conditions and some may be constitutively expressed. A variety of proteomics technologies are available for the quantitative and qualitative assessment of the protein profile under a given condition. Protein separation methods have been developed on the basis of differences in the physical and chemical properties of proteins, such as size, charge, hydrophobicity, polarity or affinity for other molecules. Many techniques for the separation of cell proteins have been described extensively in the scientific literature, including centrifugation, membrane dialysis, immunoprecipitation,

electrophoresis and chromatography (e.g. ion-exchange, size-exclusion, affinity and reversed-phase). Nonetheless, since thousands of proteins are present in any given cell system, and due to the overlapping physicochemical characteristics of some proteins, no single technology can be used to isolate all the proteins from a complex sample. Therefore multi-dimensional approaches (two or three-dimensional methods) are required for the separation.

Peptide mass finger (PMF) printing is a high-throughput tool for the identification of *C. albicans* proteins present in any public domain. The method includes mass analysis by peptide mapping and data base search. Peptide mapping is usually accomplished on MALDI-TOF mass spectrometers. In this approach, the protein sample is *in situ* digested with a specific endoproteinase (such as trypsin, which cleaves the protein at their arginine or lysine amino acids and if they are not followed by a proline). The peptide mixture is then sequentially extracted from the gel, absorbed on a metal target, co-crystallised with an acidified matrix, and ionized by laser pulses. Subsequently, an electric field accelerates these ions towards the detector, the m/z value of each ion being determined by its time of flight to travel from the source to the detector. Thus a set of protein masses are generated from the digested whole protein. Several computer algorithms (such as pepMAPPER, MASCOT, ALDENTE) programs are available for analysis of these protein masses. A vast amount of sequence information from *C. albicans* genome is now available for public use (http://www.sequence .stanford.edu/group/*Candida*) which helps further in the identification of the protein under question.

Material and Methods

3.1. Fungal Cultures

Candida albicans ATCC 10231 and its laboratory generated resistant strain AMB-R (resistant to amphotericin B) were used throughout this study. Other fungal strains used for cross reactivity studies were *C. albicans* ATCC 60193, *C. albicans* ATCC 66027, *C. albicans* ATCC 14053, *Candida parapsilosis* ATCC 22019, *Cryptococcus neoformans* ATCC 66031, *Aspergillus fumigatus* and *Trichophyton mentagrophytes.* All these fungi were grown on Sabouraud's Dextrose Agar (SDA) (Becton Dickinson, USA) slants at 35^{o}C for 24 to 72 h and stored at 4^{o}C. Bacterial strains used for cross reactivity studies were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC BAA-427*, Staphylococcus aureus* ATCC 25923*, Klebsiella pneumoniae* ATCC 27736, *Bacillus cereus* MTCC 430*.* The bacterial cultures were grown on nutrient agar (Becton Dickinson, USA) at 35^{o}C for 24h and stored at 4^{o}C. Other culture media and conditions used were Sabouraud's dextrose broth (SDB) (1% peptone and 2% dextrose), Yeast nitrogen base (YNB) (minimal media supplemented with amino acids, Sigma) with 2% dextrose, YNB with 2% N-acetyl-D-glucosamine (NAG) (Sigma) and 2% Dextrose, RPMI 1640 with 10% Fetal bovine serum (FBS) (Hybridoma tested, Sigma), DMEM with 10% FBS and serum free media (Hybridoma tested, Sigma).

3.2. Culture Conditions for *C. albicans*

The yeast form of *C. albicans* was grown in SDB or YNB (pH 4.5) in Erlenmeyer flask on a rotary shaker (200 rpm) at 28^{o}C for 16h. Alternatively, for growing *C. albicans* in hyphal form, yeast cells from late log phase culture (24-36h) were inoculated in YNB (pH 6.5) supplemented with 10% FBS at a concentration of 1x10^{6} cells/ml and incubated overnight (12-16h) at 37^{o}C without shaking. For conversion of yeast cells to hyphal form under serum free conditions, FBS was replaced with hyphae inducers such as N-acetyl-D-glucosamine. Conversion of yeast to hyphae was assessed by microscopic observation. Both yeast cells and hyphae were harvested by centrifugation at 3000 rpm for 5 min, pellets were washed three times with chilled distilled water and were either used for further experiments or stored at -80^{o}C.

3.3. Generation and Characterization of Amphotericin B Resistant Strain

3.3.1. Experimental Induction of Drug Resistance

A single colony from overnight grown culture of *C. albicans* on Sabouraud's Dextrose Agar (SDA Becton Dickinson, USA) plate at 35^{o}C was used to inoculate 5 ml of Sabouraud's Dextrose Broth (SDB, Becton Dickinson, USA) and incubated overnight in an incubator-shaker (180 rpm) at 28^{o}C. The initial minimal inhibitory concentration (MIC) of this strain was determined against amphotericin B as per guidelines of Clinical and Laboratory Standards Institute (CLSI) using SDB in 96-well tissue culture plates. Proper growth control, drug control and the negative control were adjusted onto the plate. Amphotericin B was dissolved in DMSO at a concentration of 1mg/ml and 20 µl of this was added to each well of 4th row of 96-well tissue culture plate having 180 µl SDB. From here the solution was serially diluted resulting in two-fold serial dilution of amphotericin B in subsequent wells. 100 µl of *C. albicans* (matched to 0.5 McFarland) was diluted in 10 ml of media and then 100 µl of it was added in each well. The maximum concentration of the drug tested was 50 µg/ml. The micro-titer plates were incubated at 35^{o}C in a moist, dark chamber and the MIC was recorded spectrophotometrically after 24h using microtitre plate reader containing SOFTmax Pro 4.3 Software (Molecular Devices, Sunnyvale, USA).

Initial MIC was taken as reference point for experimental induction for drug resistance. The cells of *C. albicans* (1x10^{6} cells) were inoculated in 1 ml of SDB containing AMB (½ MIC) and kept at 35^{o}C. When the cell density reached approximately 10^{8} cells/ml of culture broth, 1x10^{6} cells were transferred to fresh SDB in a test tube. For each such passage, the concentration of amphotericin B in SDB was doubled up to ~ 60 generations and the final MIC was determined. The parent strain ATCC 10231 and resultant resistant strain AMB-R were stored as glycerol stock at -80^{o}C for further studies. Stability of the resistant phenotype was tested by passaging the AMB-R in SDB (without amphotericin B) up to 60 days. Minimum inhibitory concentrations (MICs) were determined as per CLSI method described above

for both the parent and resistant strain (with/without amphotericin B) for each passage.

3.3.2. Confirmation of Resistance in Mouse Model of Candidiasis

Both the strains of *C. albicans* (parent ATCC 10231 and AMB-R) were grown over night on SDA at 35^{o}C. Cells were harvested and adjusted to 2x10^{6} cells/ml in normal saline (0.85%) using McFarland standard (0.5 OD). Simultaneously the colony forming units (cfu) were determined by 10-fold dilution and plating technique. Two groups of twelve Swiss male mice (average weight 18-20g) each were inoculated through intravenous route (lateral tail vein) with 4x10^{5} cells of parent *C. albicans* and AMB-R strain separately. The mice were then randomly subdivided into two groups of six each for parent and AMB-R strains of *C. albicans*. One group of mice for each strain served as control while in the other group the mice were given AMB (5mg/kg) orally once a day for 7 days. On day 9, the mice were sacrificed by cervical dislocation under anaesthesia. Fungal load (cfu/gm) in kidney tissues were determined by dilution and plating method as descirbed above. Prior clearance from institutional Animal Ethics Committee was obtained for all the animal experiments.

3.3.3. Time Kill Assay

Time kill assay was performed at 2xMIC, 1xMIC and ½xMIC on the basis of MICs determined for the parent *C. albicans* and AMB-R strain. Both the strains were grown on SDA slants for 24h and the cells harvested in 10 ml sterile 0.85% saline. One ml of the inoculum (matched to 0.5 McFarland standards) was added to 9 ml of RPMI 1640 medium buffered with MOPS. Amphotericin B was added at different concentration in RPMI 1640 medium as per MIC for the parent as well as the resistant strain and incubated in rotary shaker (50 rpm) incubator at 35^{o}C. One hundred µl of the broth was taken out after 0, 4, 8, 16, 24, and 48h of incubation and the cfu were determined by 10-fold dilution and plating method on SDA plates in triplicate.

3.3.4. Characterization of Amphotericin B Resistant Strain

3.3.4.1. Morphological Characterization

The morphology of yeast and hyphal form were studied by fluorescence microscopy using Calcofluor white (fluorescent brightener 28, Sigma).The cells were grown in the YNB for yeast cells and YNB+10% FBS for hyphal form and the resultant cells were pellet down and resuspended in 100 µl of Calcofluor white (1mg/ml) and incubated for 30min at room temperature. The cells were washed five times with 1xPEM (1M PIPES pH 6.9, 10mM EGTA, 10mM $MgSO_4$). After washing, the cells were resuspended in equal volume with 1xPEM. Poly-L-Lysine coated glass slides were loaded with 5 µl of cells and observed under phase contrast fluorescence microscope using appropriate filters ((excitation wavelength 340 to 360 nm, emission wavelength 400 to 440 nm) and documented.

3.3.4.2. Germ Tube Formation Assay

Germ tube formation assay was performed to study the effect of amphotericin B on yeast-to-hyphal transition in AMB-R and parent strain. The adherence of *C. albicans* germ tubes to plastic provides a simple, objective, and quantitative assay to measure the yeast to hyphal transition. Both parent and AMB-R strains were harvested from overnight grown cultures on SDA and resuspended in yeast nitrogen base (YNB) containing 10%FBS to a cell concentration of $2x10^6$ cells. AMB was added at 2xMIC to the 1^{st} well of 96 well flat bottom polystyrene plate containing 100 µl of YNB with 10%FBS and serially diluted up to 11^{th} well and the last well (12^{th} well) served as control (no drug). The plate was incubated at 37^oC for 4h and processed further using the protocol of Abe *et al* with slight modifications as follows. The wells were washed once each with 70% ethanol, 200 µl of 0.25%SDS and 3 times with distilled water to remove the non-adherent cells. The germ tubes attached to the walls were stained for 15min with 100 µl of 0.002% crystal violet in phosphate buffered saline (PBS). Excess dye was removed by washing 3 times with distilled water, once with 0.25% SDS and twice again with distilled water. The plate was dried completely and 200 µl of iso-propanol containing

0.04N HCl and 50 µl of 0.25%SDS were added to the well and mixed briefly on the orbital shaker. The absorbance at 590nm was determined on a microtitre plate reader (Molecular Devices, USA).

3.3.4.3. Dot Blot Assay for β-1,6-glucan Synthase Activity

A dot blot assay was performed to compare β-1,6-glucan synthase activity in AMB-R and its parent strain of *C. albicans* following the method of Ishiguro *et al*. with some modifications. Membrane proteins of both the strains were isolated using Mem-PER eukaryotic membrane protein extraction kit (Pierce, IL) as per manufacturer's instructions. Briefly, 15mg of exponentially grown yeast cells and germ tube of both the strains were resuspended in 80 µl of reagent A+150 mg of acid washed glass beads and vortexed for 10min. The lysed cells were mixed with 720 µl of reagent C, kept on ice and vortexed at 5 min interval for 30min. The supernatant was taken out after centrifugation at 10000 rpm for 10min and kept at 37^{o}C for 10min to separate the membrane protein fraction. Protein content of the membrane extract was measured using the Bradford assay (Bio-Rad), using BSA as a standard. For β-1,6-glucan synthase activity assay, 10 µg extracted membrane protein was mixed 2.5mM UDP-D-glucose, 150µM GTP, 1.3mM EDTA–Na, 0.75% (w/v) BSA, 4.1% (v/v) glycerol, and 100mM MES–Na at pH 6.5 and kept at 37^{o}C for 4h. For dot blot assay, 10 µl of this reaction mixture was added at different time intervals (0h, 1h, 2h and 4h) directly to the nitrocellulose membrane (Millipore, USA) and allowed to air dry at room temperature. The membrane was blocked with 3% bovine serum albumin (BSA) in Tris buffer saline (TBS) for 2h. The polyclonal antibodies generated earlier against β-1,6-glucan and β-1,3-glucan cell wall fraction were used to probe the membrane at 1:2000 dilution in 3% BSA for 1h. After washing with TBS+0.05%Tween 20 for 3 times, the membrane was further incubated with anti-mouse IgG HRP at 1:10000 dilution in 3% BSA for 1h. The blot was developed using 0.06% solution of diaminobenzedene tetrahydrochloride (DAB) in TBS containing 10 µl of H_2O_2 per 10 ml of DAB solution. The spots on the blot were then documented and analyzed on the

basis of their relative intensity using histogram function of Adobe Photoshop software.

3.3.4.4. Spectrophotometric Semi-microdetermination of Ergosterol

Total intracellular sterols were extracted by the method of Arthington-Skaggs *et.al* with some modifications. Cell cultures of both parent and AMB-R strains were grown up to stationary phase in minimal media (0.67% YNB+2% glucose), harvested by centrifugation and washed once with sterile distilled water. The net wet weight of the cell pellet was determined and vortexed for 1min in 3 ml of 25% alcoholic potassium hydroxide solution (25 g of KOH and 35 ml of sterile distilled water, brought to 100 ml with 100% ethanol). Cell suspensions were transferred to sterile borosilicate glass tubes and incubated in dry bath at 85°C for 1h. After incubation sterols were extracted by addition of a mixture of 1 ml of sterile distilled water and 3 ml of *n*-heptane followed by vigorous vortex mixing for 3-5min. The heptane layer containing total intracellular sterols was transferred to a clean borosilicate glass screw-cap tube and stored at −20°C for 24h. A 20 µl aliquot of sterol extract was diluted five-fold in 100% ethanol and scanned on a spectrophotometer (GBC 911A, Victoria, Australia) at 230 and 281.5 nm.

Ergosterol content was calculated as a percentage of the wet weight of the cell by the following equation:

$$\% \text{ ergosterol} + \% \text{ 24(28) DHE} = [(A_{281.5}/290) \times F]/\text{pellet weight},$$

$$\% \text{ 24(28) DHE} = [(A_{230}/518) \times F]/\text{pellet weight},$$

$$\% \text{ ergosterol} = [\% \text{ ergosterol} + \% \text{ 24(28) DHE}] - \% \text{ 24(28) DHE},$$

where, *F* is the factor for dilution in ethanol and 290 and 518 are the *E* values (in percentages per centimetre) determined for crystalline ergosterol and 24(28) DHE, respectively.

3.3.4.5. Expression Analysis of lanosterol 14-α- demethylase (ERG11)

The expression level of *ERG11* was checked using Reverse Transcription PCR (RT PCR) using gene specific primers and an internal control (Actin).

ERG11F TTGTTGAAACTGTCATTGATGG

ERG11R CCCAAATGATTTCTGCTGGT

ACT F TGGAATCCTGTGGCATCCATGAAAC

ACT R TAAAACGCAGCTCAGTAACAGTCCG

The total RNA from yeast and hyphae/pseudohyphae was isolated separately using RNeasy MiniKit (Qiagen, Germany) according to manufacturer's protocol. Briefly, $5x10^7$ cells of yeast and hyphae/pseudohyphae of *C.albicans* and AMB-R were harvested by centrifugation at 1000 rpm for 5min at 4^oC and 600 µl of acid washed glass beads and 600 µl RLT buffer were added. The cells were vortexed vigorously until cells were completely disrupted, centrifuged at 12000 rpm and the lysate was mixed well with equal volume of 70% ethanol. The lysate was then added to RNeasy spin column and centrifuged at 12000 rpm for 15s and flow through was discarded. The spin column was washed once with 700 µl RW1 and with 500 µl RPE buffer at 10000 rpm for 15s. To elute RNA, 30-50 µl RNase free water was added directly to the spin column membrane, centrifuged for 1min at 10000 rpm and the elute was transferred to microfuge tube for further use. The purity of RNA was checked on the basis of ratio of the readings at 260 nm and 280 nm (A260/A280). The concentration of RNA was determined by measuring the absorbance at 260 nm (A260) in spectrophotometer. After RNA quantitation using spectrophotometer, 2 µg of RNA was added to the reaction volume of 25 µl using One Step RT PCR kit (Qiagen, Germany) as per manufacturer's instructions. The 25 µl reaction mixture contained 200µM deoxynucleoside triphosphates, 0.6µM each primer, 1xPCR buffer with 1.5mM MgCl2, 2 µg of RNA sample, and 2 µl of Enzymes mix. The reaction involved reverse transcription at 50^oC for 30min, initial denaturation at 95^oC for 10min, followed by 35 cycles in series of denaturation at 95°C for 30s, annealing at 52^oC for 30s and extension at 72°C for 45s, with a final step of one cycle at 72°C for 10 min to final extension. The PCR product were checked on 2% agarose gel containing 0.5 µg/ml ethidium bromide and viewed under UV transilluminator,

documented and the band intensity were analyzed using the histogram function of Adobe Photoshop.

3.3.4.6. Extracellular Proteinase Activity Assay

Enzymatic activity of extracellular secretory aspartyl proteinases from both AMB-R and parent strains were determined. For induction of proteinase expression over night grown cells ($2x10^6$cell/ml) were inoculated in 200 ml YCB+ BSA medium (1.2% Yeast carbon base and 0.2% BSA) in Erlenmeyer flasks. The flasks were incubated for 6 days at 28^o C on an incubator shaker at 180 rpm, cfu/ml determined by dilution and plating method and then the supernatant was taken out carefully after centrifugation at 2000 rpm for 10 min. The culture supernatants were filtered through 0.22 µm pore size filter and kept at -20^oC for further use. Sap activity was determined spectrophotometrically by measuring the sample absorbance at 280nm following the degradation of the substrate (BSA) as described by Crandall & Edward (1987). A mixture of 300 µl 1% (w/v) BSA in 50mM sodium citrate, pH 3.2 and 100 µl culture supernatant was prepared. After 1 hour of incubation at 37^o C, the reaction was stopped by addition of ice cold 10% TCA. Precipitated proteins were removed by centrifugation at 5000 rpm for 30min followed by filtration through a 0.45 µm filter. The enzyme activity was measured by determining the increase in A_{280} of the supernatant as compared to blank. One unit of activity was defined as an increment in O.D. by 0.100 at A_{280}. Each experiment was carried out on three separate occasions.

3.3.4.7. Determination of Extracellular Phospholipase Activity

Extracellular phospholipase activity was determined by measuring the size of the zone of precipitation by the method of Samaranayake *et al* with some modifications. Briefly, the egg yolk medium consisted of 13.0g SDA, 11.7g NaCl, 0.111g $CaCl_2$ and 10% sterile egg yolk. The egg yolk was centrifuged at 1000 rpm for 10min at room temperature, and 20 ml of the supernatant was added to the sterilized medium. An overnight grown culture of AMB-R and parent strain on SDA was harvested and suspended in 0.9% saline to achieve 10^8 cells/ml. Five microlitre of the cell suspension were inoculated onto the

surface of the egg-yolk medium in triplicate, left to dry at room temperature and after incubation at 37°C for 48 h the diameter of the precipitation zone around the colony was determined. The experiments were repeated thrice.

3.3.5. Exposure to H_2O_2 and Menadione

The parent as well as AMB-R strains were grown overnight at 28°C in SDB under shaking conditions and the cells were harvested in exponential phase and divided into several identical aliquots. For the induction of oxidative stress, these cells were exposed to different concentrations of H_2O_2 (5, 50, 5+50mM) and menadione (0.2, 0.4, 0.8, 1.6 and 3.2mM). The viability was checked by serial dilution of the exposed cells with normal saline and plating on SDA plates. Likewise the hyphae/pseudohyphae of both the strains were exposed to same concentrations of H_2O_2 and menadione. In the experiments of acquired oxidative tolerance against H_2O_2, the cells were initially challenged with 5mM of H_2O_2 for 1h and subsequently with 50mM H_2O_2 for 2h.

3.3.5.1. Preparation of Cell Lysate

The cells exposed to different treatments as described above were harvested, washed in chilled PBS several times and resuspended in 1ml lysis buffer (100mM, pH 7.5, 1mM EDTA, 5mM DTT) containing 1xprotease inhibitor cocktail. The resuspended cells were vortexed vigorously on ice for 8 cycles of 1min each. The cell lysates were obtained by centrifugation (2000 rpm for 5 min at 4°C) and the protein contents was quantified using 2 D Quant Kit and kept at -80°C for further use.

3.3.5.2. Enzymatic Activity Assay

3.3.5.2.1. Catalase Activity

For analysis of catalase activity, the Catalase Assay Kit (Cayman Chemicals, USA) was used in accordance with manufacturer's instructions. The catalase activity was calculated by measuring formaldehyde produced with 4-amino-3-hydrazino-5-mercapto-1,2,3-triazole (Purpald) as the chromagen. The absorbance at 540 nm was read using a microtitre plate reader (Molecular Devices,) One unit is defined as the amount of enzyme that causes the

formation of 1.0 nmol of formaldehyde per minute at 25°C. The following formula was used to determine catalase activity.

$$\text{CAT activity} = \frac{\mu M \text{ of sample}}{20min} X \text{ sample dilution} = nmol/min/ml$$

3.3.5.2.2. Glutathione peroxidase (GPx) activity

GPx activity was calculated using Glutathione Peroxidase Kit (Cayman Chemicals, USA) according to manufacturer instructions. The Glutathione peroxidase activity was calculated by a coupled reaction with glutathione reductase. Oxidized glutathione, produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340nm. The reaction rate at 340nm can be determined using NADPH extinction coefficient of 0.00373 μM^{-1}. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0nmol of NADPH to $NADP^+$ per minute at 25°C.

$$\text{GPx Activity} = \frac{\Delta A340/min}{0.0373\mu M} x \frac{0.19ml}{0.02ml} x \text{Sample dilution} = nmol/min/ml$$

3.3.5.2.3. Glutathione-S-Transferase Activity Assay

The Glutathione-S-Transferase (GST) Assay Kit (Sigma, USA) was used as per manufacturer instruction. The kit is based on utilization of 1-chloro-2, 4-Dinitrobenzene (CDNB), suitable for various GST isozymes. Upon conjugation of the thiol group of glutathione to the CDNB substrate, the increase in the absorbance at 340nm is directly proportional to the GST activity.

$$\text{GST activity} = \Delta A340/min x V(ml) x \text{ dil. of sample } / \varepsilon mM x Venz(ml) = nmol/ml/min$$

3.3.6. Characterization of AMB-R at Genetic Level

3.3.6.1. DNA Isolation

3.3.6.1.1. Phenol: Chloroform Method

Both the parent and AMB-R strains grown in SDB at 28°C were harvested by centrifugation at 5000 rpm for 5min. The cell pellet was suspended in STES buffer (0.2M Tris-HCl pH 7.6, 0.5M NaCl, 0.1% w/v SDS, 0.01mM EDTA), and acid washed glass beads (425-600µm) were added to it in a ratio of 1:1 and

vortex vigorously for 30min with intermission after every 5min to avoid heating. Cell debris was removed by centrifugation and supernatant treated with phenol: chloroform: isoamylalcohol (25:24:1). Aqueous phase was carefully taken out and treated with 10 µg RNase A and 100 µg proteinase K. DNA was precipitated by adding equal volume of iso-propanol and the pellet was stored in 1xTE buffer after washing with 70% ethanol at –20^{o}C.

3.3.6.1.2. DNeasy Protocol

Both the strains were inoculated in 100 ml SDB under shaking conditions at 37^{o}C to obtain log phase cultures. Microscopic examination was done to test the purity of the cultures, and the cells were harvested by centrifugation at 6000 rpm for 15min at 4^{o}C. The pellets were washed twice with 0.8% physiological saline and transferred to 200 µl of extraction buffer (0.2M Tris-Cl pH 7.6, 0.5M NaCl, 0.1% SDS, 0.01M EDTA). Glass beads (425-600µm**)** were added (1:1) to this mixture and vortexed vigorously in a bead beater (HamiltonBeach/Proctor-Silex, Inc., USA) to achieve 60% lysis of the cell mass. Fungal DNA from cell lysate was recovered with a DNeasy® plant mini kit (Qiagen, Germany) according to the manufacturers' instructions. Briefly 200 µl of lysate was mixed with lysis buffer AP1, vortexed and incubated for 10 min at 65^{o}C followed by addition of 130 µl buffer AP2 to the lysate and incubated for 5min on ice. The lysate was applied to QIAshredder mini column and centrifuged for 2min at 14000 rpm. Buffer AP3/E was added to the lysate in a ratio of 3:2. After gentle shaking, mixture was passed through DNeasy mini column and the flow through was discarded. Then the column was washed with buffer AW1 and AW2 and the bound DNA was eluted with AE buffer by centrifugation at 8000 rpm for 2min and the collected eluents were stored at –20^{o}C.

All the samples were electrophoresed on 1% agarose gel with 1xTBE buffer (8.9mM Tris-borate, 0.2mM EDTA), and analyzed after staining with ethidium bromide. The purity of extracted DNA was checked at 260/280nm (UV/VIS 911A, GBC Scientific Equipments, Australia).

3.3.6.2. PCR Amplification and Sequencing

The extracted DNA was subjected to amplification using thermal cycler (Helena Biosciences, U.K.). All the primers (MWG Oligo), provided in lyophilized form were dissolved in autoclaved TDW to make 100 µM stock solutions. To further use, these primer pair solutions were diluted to 10µM working solution in sterilized TDW.

Cytochrome P-450 lanosterol 14-alpha-demethylase (ERG11)

PCR was standardized using ERG11 primers. The sequences of the primers used for the reaction was

ERG11F TTGTTGAAACTGTCATTGATGG

ERG11R CCCAAATGATTTCTGCTGGT

A 25 µl reaction mixture contained 100µM dNTPs, 0.1µM each primer, 1x PCR buffer with 1.5mM $MgCl_2$, 2 µl of template DNA sample, and 1U *Taq* polymerase (Qiagen, Germany). The reaction involved initial denaturation at 95^{o}C for 10min followed by 35 cycles in series of denaturation at 95^{o}C for 60s, annealing at 50^{o}C for 60s, and extension at 72^{o}C for 90s, with a final step of one cycle at 72^{o}C for 10min to final extension.

CSP37

Another PCR reaction was standardized using CSP37 primers. The sequences of the primers used in the reaction was as follows

CSPF CAGCCATGGTCTGCTGGAAA

CSPR ACGGTTAAACAAATCGTCAAA

The reaction mixture and conditions were the same as those used above with the exception that the annealing temperature was 52^{o}C.

3.3.6.3. PCR Product Purification

The amplified PCR products were purified using Montage DNA gel extraction kit (Millipore, USA). The PCR products were loaded onto <1.25% agarose gel prepared in 1x modified TAE supplied with the kit and kept for electrophoresis

in submarine tank at 50mA. After 30min, the gel was observed under UV transilluminator and the PCR product band was excised from the gel using sharp edge blade. The piece of gel was loaded on supplied Montage DNA gel extraction column and was centrifuged at 8000 rpm for 10min. The purified PCR product was collected and kept at -20^{o}C for further use.

3.3.6.4. Sequencing

Purified PCR products were subjected to identification by partial sequencing using one of the primers through ABI prism automated DNA Sequencer (Model 3100 version 3.0: Applied Biosystems, U.K.) in DNA sequencing facility, South Campus, Delhi University. Successfully obtained clean data of up to 500bp length was matched with available database BLASTn (www.ncbi.nlm.nih.gov/BLASTn). Maximum matched sequences were aligned with obtained sequence through CLUSTALW, a database for alignment for DNA and protein sequences (www.ebi.ac.uk/clustalw).

3.4. Isolation of *C. albicans* Yeast and Hyphal Cell Wall

Cells of both the morphological forms of *C. albicans* stored at -80^{o}C were thawed and the pellet was resuspended in chilled 20mM Tris-Cl pH 7.4, supplemented with protease inhibitor cocktail (to a final concentration of PMSF 1mM, EDTA 1mM, AEBSF 1mM, 1, 10-phenanthroline 5mM, pepstatin A 20 µM, E-64 10 µM), to a cell density of 1x10^{8}cells/ml. To 10 ml of this cell suspension, an equal amount of acid washed glass beads were added and vortexed vigorously in the jacketed mixing chamber of bead beater (Hamilton Beach/Proctor-Silex, Inc.) that was cooled with glycerol at -20^{o}C. The instrument was operated with cycles of mixing for 1min followed by a pause of 2min. After a total of 10-12 cycles, the lysate was observed under phase contrast microscope and the cell wall was pelleted by centrifugation. The cell wall pellet was extensively washed with chilled triple distilled water supplemented with PMSF and benzemidine hydrochloride to a final concentration of 1mM each, and stored at –80^{o}C. For cross reactivity studies, cell walls of resistant strain AMB-R, *C. parapsilosis*, *C. tropicalis, C.*

neoformans, *A. fumigatus* and *T. mentagrophytes* were similarly isolated and stored.

3.5. Isolation of Covalently Linked Proteins

The isolated cell wall was treated with different chemicals in order to isolate covalently linked cell wall proteins.

3.5.1. HF-Pyridine Release of GPI Cell Wall Proteins

In order to isolate GPI cell wall proteins (GPI CWPs), cell wall of parent *C. albicans* and AMB-R strain was treated with HF-Pyridine (50%v/v, Aldrich) at 4^{o}C overnight. After overnight incubation the reaction was stopped with equal volume of ice-cold triple distilled water. The treated fraction was dialyzed against distilled water for 48h to remove HF-Pyridine. The dialyzed protein fraction was freeze-dried and kept at –80^{o}C.

3.5.2. Enzymatic Release of GPI Cell Wall Proteins

The isolated cell wall of both parent *C. albicans* and AMB-R was washed extensively using wash buffer (50mM Tris-Cl pH 7.4, 1mM PMSF). After washing, the cell wall was resuspended in 2 ml of extraction buffer (1500U lyticase/g of cell wall extract, 50mM Tris-Cl pH 7.4, 10mM DTT, and 1mM PMSF) and was incubated at 37^{o}C for 16h with gentle shaking. The enzymatic reaction was stopped using 10% SDS at 0.4% final volume and heating at 100^{o} C for 5min. The treated cell wall was centrifuged for 10min at 4000 rpm. The supernatant were taken and kept at -80^{o}C.

3.6. Protein Estimation Using 2D Quant Kit

Protein estimation was done using 2D Quant kit (GE health care, USA) throughout this study. Estimation of the protein in the samples was carried out in 2 ml microfuge tubes as per manufacturer's instruction. Each test protein sample was taken in duplicate in 2 ml microfuge tubes. To each tube 500 µl of precipitant provided in the kit was added and vortexed briefly for 2-3min at RT. To this 500 µl of co-precipitant was added and mixed briefly by vortexing and the tubes were centrifuged at 10000 rpm for 5min at room temperature. The pellets thus obtained were resuspended in 100 µl of copper solution using a

micropipette. To each tube 400 µl of TDW was added and mixed. A colour reagent provided in the kit was prepared by mixing 100 parts of colour reagent-A with 1 part of colour reagent-B. To each tube, 1 ml of this colouring reagent was added and incubated at room temperature for 20min. After incubation 200 µl of solution from each tube was taken in microtitre plate and the absorbance was read at 480 nm in a microtitre plate reader while taking TDW as a blank. The protein quantity was estimated by Versamax® software using the standard curve generated.

3.7. Raising Polyclonal Sera in Mice against GPI Cell Wall Proteins of *C. albicans*

The GPI cell wall proteins stored at -80^{o}C was thawed and 100 µl of this was suspended in 200 µl of PBS. The protein value of this suspension was determined as described above and the suspension was then diluted in PBS, adjusting protein content to 1mg/ml. This suspension was mixed vigorously with equal volume of Freund's complete adjuvant using a three-way valve just before administration. The Swiss mice were allowed to acclimatize under laboratory conditions for one week and 200 µl of blood was drawn through tail vein from each animal and the sera was separated and stored at -20^{o}C in aliquots. This serum served as zero hour control in ELISA and immunoblotting experiments. The GPI cell wall protein was emulsified with Freund's complete adjuvant (500 µl, 1:1) and 200 µl of this was injected subcutaneously in 5 mice each. After 15-20 days, when the pustules formed by the injection have disappeared the mice were given two booster doses of cell wall fraction with equal volume of Freund's incomplete adjuvant. After one month since the last booster, 100-200 µl of blood was collected from the tail vein and the serum collected was cleared by centrifugation, aliquoted and stored at -80^{o}C.

3.8. SDS PAGE of Covalently Linked Cell Wall Proteins

The yeast and hyphal covalently linked cell wall proteins were resolved by SDS PAGE and subsequently the resolved protein bands were electro-transferred on to a nitrocellulose paper (NCP) as follows.

SDS PAGE

The protein sample of yeast and hyphal covalently linked cell wall proteins of *C*. *albicans* were mixed and boiled in equal volume of SDS sample loading buffer (125mM Tris-HCl, 2% SDS, 5% β-ME, 10% glycerol, and traces of bromophenol blue) for 5min and centrifuged for 5min at 12000 rpm. After estimating the protein values, SDS PAGE was performed using Laemmili's discontinuous two gel system to resolve the proteins. For SDS PAGE, 12% resolving gel and 4% stacking gel was used throughout the study. The percentage of the gel was determined by the acrylamide percentage. The stock solutions used for SDS PAGE were 30% acrylamide (29.3% acrylamide and 0.7% bis-acrylamide), 1.5 M Tris pH 8.8, 0.5 M Tris pH 6.8, 10% SDS, 10% ammonium persulfate (APS) and TEMED. After polymerization of resolving gel, the mixture of stacking gel was poured on top of it and a comb placed to form the wells. A pre run of the gel was carried out (10 mA, 30min), the samples were loaded (20 µg/well of protein value) and electrophoresis was carried out at a constant current of 10 mA till the dye front reached the bottom of the gel and subsequently, the gel was placed in transfer buffer for equilibration for 15min.

3.9. Electro-transfer of Proteins

After SDS-PAGE, the gel was equilibrated in transfer buffer for 15-25min along with a piece of nitrocellulose paper (NCP) covering the gel. The gel along with the NCP was placed inside the blotting cassette keeping NCP towards the anode. The blotting apparatus filled with transfer buffer and 100 mA current was applied for 1-2h. After 3h, the blotted NCP was taken out from the cassette, rinsed once with TDW and allowed to air dry. To visualize the protein transfer efficiency, the blot was stained with Ponsceau S dye (Sigma), and destained to optimum background with TDW. The blot was then allowed to air dry and the protein profile was documented on a conventional document scanner.

3.10. Immunoblotting Using Pooled Patient Sera and Antiserum Raised in Mice

After destaining, the NCP blot was immersed in 5% low fat skimmed milk blocking solution prepared in TBST(20mM Tris-HCl pH 7.6, 0.8% NaCl,0.05% Tween 20) for 90min on a shaker to block non specific binding site on membrane. Pooled patient serum or polyclonal raise in mice were used as primary antibody in appropriate dilution (1: 5000) prepared in TBST. The blot was kept in primary antibody for 90min and was washed with four changes of TBST, 5min each under shaking condition. The NCP was kept in a solution of anti-human or anti-mouse IgGAM-HRP at a dilution of 1: 10000 in TBST and kept on a rocker with mild agitation for 90min. The NCP was washed again with three changes of TBST, 5min each, and two more changes in TBST without Tween 20 (TBS). The immuno-reactive proteins were revealed by developing the blot with a 0.06% solution of diaminobenzedene tetrahydrochloride (DAB) in TBS containing 10 µl of H_2O_2 per 10 ml of DAB solution. After the color development reached to optimum intensity, the reaction was stopped by washing the blot with TDW. The blot was then allowed to air dry and documented.

3.11. Identification and Characterization of GPI CWPs Proteins by MALDI-TOF

Matrix assisted laser desorption and ionization time of flight (MALDI-TOF) was used for identification of immunogenic proteins detected on SDS-PAGE. Immunogenic proteins were first marked on the gel images and high-resolution printouts of different segments of gel were taken. The protein bands of interest and a “control” piece of gel from blank region were excised manually and immersed quickly 100-200 µl of TDW in a labeled low binding polypropylene microfuge vial and kept at -20^{o}C until used further.

Depending upon the stain used the gel pieces were destained and treated before proceeding for trypsinization. For Coomassie stained gel pieces, the gel pieces were taken out from -20^{o}C, thawed, and water was replaced with 200 µl of 100mM ammonium bicarbonate (ABC) made in 50% acetonitrile

(ACN), and allowed to shake for 30min. The ABC solution was replaced with fresh solution and allowed to rock for another 30min (for remover of stain) and if required the process was repeated once more, otherwise the gel pieces were dehydrated by addition of 200 µl of 100% ACN, which was replaced again after 5min. The gel pieces were further dried on vacuum centrifuge (CENTRA-VAC, Korea) for 1h. A highly purified trypsin (20ng/vial), optimized for peptide mass fingerprinting (Sigma, USA) was first dissolved in 100 µl of 10mM HCl and then 900 µl of 100mM ABC prepared in 9% ACN was added to it. The vials containing vacuum dried gel pieces were kept on ice and 20-50 µl of trypsin of final concentration (20ng/ml) was added to it. The gel pieces were allowed to hydrate on ice for about 30min and the extra trypsin was carefully removed. Then few microliters of 100mM ABC were added to cover the gel pieces that prevented the drying during incubation and the gel pieces were shifted to a 37^{o}C incubator for 4h to overnight. After the incubation was over, the reaction of trypsin was stopped by addition of 1% trifluoroacetic acid (TFA) such that the pH of the reaction mixture was 2. The microfuge tubes were briefly spun and the supernatant was collected in a fresh, siliconized, low binding microfuge tube. To these gel pieces 100 µl of 50% ACN, 0.2% TFA was added and kept on a shaker or sonicator bath for 30min. The tubes were again briefly spun and the supernatant was pooled. The pooled supernatant was then reduced to 2-3 µl by evaporating on a vacuum centrifuge and stored at -20^{o}C until further analysis.One microlitre of the above concentrated sample was mixed with 1 µl of matrix solution (10% α-cyano-4-hydroxycinnamic acid) and 1 µl of this was plated onto MALDI target plate. The sample was allowed to air dry and then plate was carefully fitted on to the robotic arm of the machine. The instrument (Micromass TOF) was operated in reflectron mode, with delayed time of extraction. All mass spectra recorded were externally calibrated with a set of highly purified synthetic peptides (Sigma, USA). For internal calibration, the matrix and trypsin autolysis product peaks were used. Calibration was also performed every time before the samples were analyzed. Each spectrum was an average of a number of spectra collected over a given period.

Protein identification was carried out by using the following softwares

MASCOT (www.matrixscience.com)

ALDENTE (www.expasy.org/tools/aldente)

pepMAPPER (www.nwsr.manchester.ac.uk/mapper)

Initial search parameters were as follows: Cys as *S*-carbamidomethyl derivative and Met in oxidized form, one missed cleavage site, and peptide mass tolerance of 0.3 Da. No filter was placed on species, molecular weight, or pI of protein. Other search parameters were taken as default setting. Initially, all mass values in the spectra were fed in the search. If this resulted in large number of hits, then the data was manually edited by subtracting the trypsin autolysis peaks, the peaks that were common with other spectra of the same batch, and all peaks that were present in the control samples.

3.12. GPI CWPs and Macrophage Interaction

3.12.1. Macrophage Culture

Macrophage cell line J774 was used for the interaction studies. The macrophage cell line was maintained in DMEM+10%FBS in a CO_2 incubator. The cells were sub-cultured on regular basis and when the cell density reached 70% they were harvested by scraping with rubber scrapper, centrifuged, and inoculated into fresh media at final cell density to$1x10^6$ cells/ml.

3.12.2. DNA Fragmentation Assay

To check the effect of cell wall fraction on macrophage viability, DNA fragmentation assay was performed. J774 cells were sub-cultured in 24 well tissue culture plate one day prior to the experiment. On the day of experiment, GPI CWPs of both hyphae and pseudohyphae forms of *C. albicans* and its resistant strain AMB-R were incubated with J774 cells at different concentrations. After 16h incubation, macrophages were harvested using a cell scraper and centrifuged at 1000 rpm for 10min at room temperature. The macrophage cell pellet was incubated for 1h at 50^oC in hypotonic cell lysis

buffer (10mM Tris-HCl pH7.4, 10mM EDTA, 1%TritionX100, 100 µg/ml Proteinase K). After incubation, the cell lysates were clarified by centrifugation at 1000 rpm for 30min at room temperature. The clarified supernatant were mixed with phenol: chloroform: isoamyl alcohol (25:24:1) equal volume and centrifuged at 10000 rpm for 5min. The aqueous layer was taken our carefully and mixed once with chloroform: isoamyl alcohol and centrifuged at 10000 rpm for 5min. The final aqueous layer was mixed with 50% iso-propanol+0.5M NaCl and incubated at -20^{o}C overnight. The precipitate was pelleted down by centrifugation at 12000 rpm at 4^{o}C for 30min, washed once with 70% ethanol, air dried, and dissolved in 20 µl of 1xTE buffer. The isolated DNA were run on 2% agarose gel and documented.

3.12.3. Cytokine Level in Macrophages

Macrophages were incubated as described above with GPI cell wall proteins of yeast and hyphal form of *C. albicans* and its resistant strain for cytokine level analysis. After incubation for 4h, 2x10^{6} macrophage cells were washed twice with chilled DEPC treated PBS and mixed with monophasic agent 1 ml TRIzol® reagent (Invitrogen life technologies, USA) and incubated for 5min at room temperature for complete dissociation of nucleoproteins. After incubation 200 µl of chloroform: isoamyl alcohol (1:1) was added to it and centrifuged at 12000 rpm for 5min. The aqueous phase was carefully taken out and 0.25 ml of RNA precipitate solution and 0.25 ml of iso-propanol were added to the mixture and incubated overnight for RNA precipitation at –20^{o}C. After this, the mixture was centrifuged at 14000 rpm for 15min and the supernatant was carefully removed. The pellet was washed with 70% ethanol and finally dissolved in DEPC treated water.

The extracted RNA was checked on formaldehyde gel in MOPS buffer. Agarose gel (1.5%) was prepared in 1xMOPS buffer containing 2.2M formaldehyde. Two microlitre of extracted RNA was mixed with 10 µl formamide, 4 µl formaldehyde, and 2 µl of 1mg/ml ethidium bromide and heated at 85^{o}C for 15min and loaded onto the gel with 2 µl of loading dye. The RT-PCR RNA was treated with DNAse before proceeding for complete

removal of DNA. The RNA (20 µl) sample was mixed with 1xDNAse buffer and 10U of DNAse and incubated at 37^{o}C for 15min followed by addition of 10 µl stop solution. These samples were then heated at 85^{o}C for denaturation of enzyme and RNA. Then RNA was quantified using Gene Quant (Amersham Biosciences) and diluted accordingly in DEPC treated water to get a final concentration of 10ng/ml of RNA samples.

RT PCR

RT-PCR reactions for IL10, iNOS, IL-12, MIP-2, and β-actin were performed by using components of Qiagen one step RT-PCR kit. The 25 µl reaction mixture contained 200µM deoxynucleoside triphosphates, 0.6µM each primer, 1xPCR buffer with 1.5mM MgCl2, 2 µg of RNA sample, and 2 µl of Enzymes mix.

1. IL-12:

The primer pair for the IL-12 was:

CTGGCCAGTACACCTGCCAC

GTGCTTCCAACGCCAGTTCA

The conditions for IL-12 were reverse transcription at 50^{o}C for 30min, initial denaturation at 95^{o}C for 10min, followed by 35 cycles in series of denaturation at 95°C for 30 s, annealing at 66^{o}C for 30 s and extension at 72°C for 30 s, with a final step of one cycle at 72°C for 10min to final extension.

2. MIP-2:

The primer pair for MIP-2 was;

GAACAAAGGCAAGGCTAACTGA

AACATAACAACATCTGGGCAAT

The reaction involved reverse transcription at 50^{o}C for 30min, initial denaturation at 95^{o}C for 10min, followed by 35 cycles in series of denaturation at 95°C for 30 s, annealing at 56^{o}C for 30 s and extension at 72°C for 30 s, with a final step of one cycle at 72°C for 10min to final extension.

3. β actin, IL10, iNOS:

The primers for β actin were;

TGGAATCCTGTGGCATCCATGAAAC

TAAAACGCAGCTCAGTAACAGTCCG

The primers of IL10 were;

CTATGCTGCCTGCTTACT

TCACTCTTCACCTGCTCCAC

The primers for iNOS were;

TCTGCGCCTTTGCTCATGAC

TAAAGGCTCCGGGCTCTG

The reaction involved reverse transcription at 50^{o}C for 30min, initial denaturation at 95^{o}C for 10min, followed by 35 cycles in series of denaturation at 95°C for 30sec, annealing at 56^{o}C for 30sec and extension at 72°C for 45 sec, with a final step of one cycle at 72°C for 10min to final extension. The condition for iNOS and IL10 were similar to β-actin except for the annealing temperature. The annealing temperature for iNOS and IL10 was 55^{o}C and 60^{o}C respectively.

The amplified products were checked on 1% agarose gel electrophoresis.

3.13. Generation of Hybridomas and Production of Monoclonal Antibodies

3.13.1. Cell Line

Mouse myeloma cell line (Sp2/O) was obtained from National Institute of Immunology, New Delhi and maintained in RPMI containing 10% FBS at 37^{o}C and 5% CO_2 atmosphere. It was also cryopreserved (10% DMSO, 40% RPMI 1640 and 50% hybridoma tested FBS) in liquid nitrogen in aliquots for future use as well.

3.13.2. Mice

Female BALB/c mice were used throughout this study. All the animals were maintained and cared in the Laboratory Animal Facility of the institute according to the norms laid down by the animal ethics committee. Prior clearance from the animal ethics committee was obtained before performing any experiment on animals.

3.13.3. Immunization of Mice

Ten BALB/c mice were allowed to acclimatize to the laboratory environment for 5-7 days. The mice were immunized subcutaneously with 100 μl of hyphal GPI cell wall proteins as antigen (100 μg/dose) preparation in Freund's complete adjuvant (1:1). This was followed by three consecutive booster doses at 15 days interval, subcutaneously with the same antigen in incomplete Freund's adjuvant and the mice were observed daily for secondary infection if any. After 30 days of the last booster dose, 20-50 μl of blood was drawn from the tail vein of each animal to checking the optimum immunization by performing ELISA and Western blot. Two to three mice that had the highest antibody titre were selected for fusion experiments. The mice selected for fusion experiment were given a final booster dose of 100 μl (1mg/ml) of hyphal GPI protein fraction cell wall (without any adjuvant) intraperitoneally, 5-6 days prior to fusion experiment.

3.13.4. Sub-culturing and Maintenance of Sp2/O

The cell line Sp2/O was essentially grown and maintained by regular culture on every fourth day in RPMI 1640, supplemented with 15mM L-glutamine, 200mM sodium bicarbonate, 10% FBS (Hybridoma tested, Sigma, USA), 50 U/ml ampicillin, 25 μg/ml streptomycin, 500 μg/ml gentamicin, and 8 μg/ml amphotericin B. As a rule, the sub culturing was performed by dislodging the cells from a confluent flask and seeding $1x10^6$ cell/ml of this cell suspension into 5 ml of fresh medium in tissue culture flask.

For fusion experiments, sufficient number of Sp2/O cells ($3x10^9$) was harvested from exponential phase (36h) of cell culture grown in RPMI 1640.

Prior to expansion, Sp2/O cells were checked for their selectivity on HAT (final concentration in media, 0.1mM hypoxanthine, 4μM aminopterin, and 16μM thymidine) medium. This was done by sub-culturing an aliquot of cells in the RPMI media supplemented with HAT.

3.13.5. Preparation of Spleen Cells and Myeloma Cells for Fusion

Immunized mouse with highest antibody titer was sacrificed through cervical dislocation on the day of fusion experiment. Spleen was taken out aseptically in a sterile petri dish, washed with 5 ml of incomplete RPMI media (without FBS) and transferred to another petri dish. To this 5 ml incomplete RPMI media 1640 (without FBS) was added and the spleen was disintegrated with a sterile plunger on a sterilized sieve to release single cell suspension or splenocytes. Further, small pieces and debris of the spleen were further passaged through pippette in incomplete RPMI 1640 media few times to obtain more number of single cell splenocytes. The cell suspension was collected in a 50 ml centrifuge tube and centrifuged at 1200 rpm for 5min. The supernatant was removed and the cell pellet was resuspended in 10 ml of fresh medium and kept on a water bath at 37^{o}C until for 5min.

The Sp2/O myeloma cells were dislodged and collected aseptically in 50 ml centrifuge tube. The cells were centrifuged at 1200 rpm for 5min and the supernatant was taken out for further use. The Sp2/O cell pellet was washed twice with incomplete RPMI media to remove FBS sticking to the cells and the cell pellet was kept at 37^{o}C.

3.13.6. Fusion of Splenocytes with Sp2/O Cells

The pellet obtained by harvesting of splenocytes and SP2/O cells were resuspended in incomplete RPMI media and mixed in 1:2 ratio together. The mixture was centrifuged first at 1200 rpm for 5min and the supernatant was removed completely using a pipette, without disturbing the pellet. The tube containing pellet was equilibrated for 3-4min at 37^{o}C on a water bath. To this pellet, 1 ml of PEG/DMSO solution (50% polyethylene glycol and 10% DMSO) already equilibrated to 37^{o}C was slowly added to the suspension with gentle swirling of over a period of 1min and the mixture was allowed to stand for one

minute. To this suspension, 5 ml of incomplete RPMI 1640 was added gradually with gentle swirling of the tube at 37^{o}C for 3-5min on water bath followed by 10 ml of the same media as above and PEG/DMSO was diluted immediately by adding RPMI 1640 containing 10% FBS up to 50 ml. The 50 ml cell suspension was then transferred to a tissue culture flask containing 150 ml of RPMI 1640 supplemented with 10% FBS, 50 ml of Sp2/O cell culture supernatant and HAT supplement (final concentration, 0.1mM hypoxanthine, 4µM aminopterin, and 16µM thymidine). The content of the flask were distributed in aliquots of 200 µl/well in 96 well tissue culture plates and incubated in an atmosphere of 5% CO_2 at 37^{o}C in an incubator. To confirm proper fusion, 10-20 µl of this cell suspension was placed on a glass slide and observed under microscope. Each well of these plates was observed carefully from day 2 to rule out contamination if any and to check the hybrid cell multiplication. Wells that appeared contaminated were marked and later sterilized by removing carefully all the contents of the well and adding 200 µl of sodium azide (0.1 mg/ml). Three days later few cells appeared alive and started dividing. At this stage, each well was supplemented with 3xHT supplement to attain a final concentration of 0.1mM of hypoxanthine and 16 µM of thymidine. The cells were grown further for 3 more days and then the screening for positive hybridoma clones was started.

3.13.7. Screening of Positive Hybridomas

Screening for antibody producing hybridoma clones was started after 7 days of incubation when the clones started looking like a bunch of grapes.

Screening by ELISA

Initial screening for antibody producing hybridoma cells was done by Enzyme linked immunosorbent assay (ELISA). For the screening, 96-well flat bottom ELISA plates (Greiner Bio One GmbH) were coated with 50 µl of hyphal GPI CWPs adjusted to a concentration of 50 µg/ml in 0.6M bicarbonate buffer (pH 9.6) and incubated for 90min at 37^{o}C or overnight at 4^{o}C. The antigen coated wells were blocked by adding 150 µl of 1% (w/v) bovine–serum albumin in PBS for 90min at 37^{o}C followed by washing of the wells once with PBS-T20 (5

min). After blocking, 50 µl of cell culture supernatant was aseptically poured directly into antigen coated ELISA plates using an 8 channel pipette such that the ELISA plate was an exact replica of culture plate. Care was taken not to spill contents while pipetting and the pipette tips were changed for every well to avoid cross contamination from other monoclonal antibodies. The plates were incubated for 90min at 37^{o}C, washed thrice with PBS T-20 (5min each) and were incubated with peroxidase-conjugated anti-mouse IgG, IgA and IgM diluted 1:5000 (each) in PBS T-20 for 90min at 37^{o}C. After washing, 100 µl of substrate containing 0.05% (w/v) O-phenylene diamine dihydrochloride (OPD) (Sigma) and 0.03% H_2O_2 (v/v) in phosphate citrate buffer 0.15M (pH5.0) were added to each well and the plates were incubated in dark at room temperature for 30min. The reaction was stopped with 10 µl of 7% H_2SO_4 and optical densities were read on a micro titre plate reader (Molecular Devices, USA) at 450 nm.

3.13.8. Single Cell Cloning of Positive Hybridoma Clones

Positive hybridoma clones identified earlier on the basis of ELISA were sub cloned by limiting dilution method to reach single cell per well in 96-well culture plates. When the population of wells containing single cell were reached to a size of at least 200 cells per well, 100 µl of culture supernatant was removed to perform ELISA. The ELISA positive colonies were again subjected to a second round of limiting dilution followed by selection through ELISA. The hybridoma clones positive for antibodies in ELISA were adopted for 24-well followed by 6-well cell culture plates. After the wells have become confluent, the cells from 6-well cell culture plates were collected and transferred to a 50 ml tissue culture flask in 7 ml of media to increase the volume of antibody producing hybridoma clones.

3.14. Western Blotting Using GPI CWPs

The ELISA positive hybridoma clones consistently producing monoclonal antibodies with very high titre were grown in 24 well tissue culture plates and their supernatant was used for Western blot studies. SDS PAGE was performed to resolve the hyphae GPI CWPs and the resolved protein bands

were transferred on to a nitrocellulose paper (NCP) as described earlier. The NCP was then carefully cut into strips of individual lanes and numbered before processing further. These individual strips were blocked using 5% fat free milk solution in Tris buffered saline (TBS, pH 7.4) for 1h at 37^{o}C. The strips were washed once with TBS containing 0.05% Tween 20 to remove excess blocking reagent and the cell culture supernatant from single cell clones (ELISA positive) was added and incubated for 1h at 37^{o}C. The blots were then washed thrice with TBS-T20, 5min each, probed with 1:10000 dilution of secondary antibody (peroxidase-conjugated anti-mouse IgG, IgA, and IgM polyclonal antibody raised in rabbit), washed again, developed with DAB, and documented.

3.15. Production of Monoclonal Antibodies under Serum-free Conditions

To avoid the presence of serum in medium containing monoclonal antibodies the hybridoma cells were first acclimatized to grow in serum free medium (serum-free low protein hybridoma medium' Sigma, USA, without any antibiotics) by gradually diluting the culture medium with increasing concentration of serum free medium. Initially, for 3 ml of hybridoma cell suspension in complete RPMI 1640 media, 4 ml of serum-free medium was added in 50 ml tissue culture flask and kept at 37^{o}C in a CO_2 incubator. After 2-3 days when media appeared orange to yellow, the cells were dislodged and cultured in fresh serum free medium by reducing the volume of complete RPMI 1640 media gradually. After adaptation in serum free media, the hybridoma clones were expanded by sub-culturing in 10 flasks and incubated in a CO_2 incubator for 4 days. After 4 days, the supernatant was collected and the flasks were replenished with fresh serum free medium. The serum free supernatant containing monoclonal antibodies were centrifuged, filtered through 0.22 µm membrane filter and preserved at -20^{o}C.

The supernatant for each monoclonal antibody was precipitated with 50% ammonium sulphate (313g/L) in flasks at 4^{o}C avoiding any accumulation of crystals. These flasks were left at 4^{o}C for 16h and the precipitate was collected by centrifugation (12000 rpm for 30min) and reconstituted in minimal

amount of PBS. The reconstituted antibody solution was dialysed using Mini dialysis kit (Amersham, USA) at 4^{o}C, against PBS with 5 changes within duration of 14-16h to completely remove the salt. After dialysis the solution containing monoclonal antibodies was centrifuged at 12000 rpm and supernatant was collected in a sterile labelled microfuge tube. The protein content (antibody concentration) in the solution was estimated by 2D Quant kit and stored at -20^{o}C until used further.

3.16. Cryopreservation of Positive Clones

Positive hybridoma clones were cryopreserved for long term storage as follows. The clones were sub-cultured a day before cryopreservation, harvested and the cell count & viability was assessed using hemacytometer and trypan blue. The cells were then pelleted by centrifugation at 1000 rpm for 5min at room temperature and resuspended in chilled cryopreservation mixture containing 10% DMSO, 40% RPMI 1640 and 50% hybridoma tested FBS to a concentration of 1x10^{6} cells/ml. The cell suspension in aliquots of 1 ml was distributed in labelled cryovials and immediately kept in a cryopreservation box that contained Iso-propanol (at 4^{o}C). The box was kept at -80^{o}C overnight and transferred to liquid nitrogen container.

3.17. Iso-typing of the Monoclonal Antibodies

Mouse monoclonal antibody Iso-typing kit (ISO-2, Sigma) and Isostrip Iso-typing kit (Santa Cruz Biotechnology, Inc) were used to identify the isotype of the monoclonal antibodies produced by the hybridoma clones in the study.

Mouse monoclonal antibody Iso-typing kit is based on Capture ELISA method. In this method, Iso-type specific antibodies were diluted 1:1000 in PBS and 100 µl of each diluted antibody was added into each well of the ELISA plate in replicates and incubated at 37^{o}C for 1h. The coating solution containing antibodies was removed and the plate was washed thrice (5min each) with PBST (PBS with 0.05% Tween-20). The test monoclonal antibody (MAb) in 100 µl PBS were put in each well and incubated at 37^{o}C for 1h and were washed three times (5min each) using washing buffer. One hundred microlitre of peroxidase conjugated goat anti-mouse IgG (Fab specific) antibody (diluted

1:600 in PBST) was added into each well and incubated at 37^{o}C for 30min. After 30min the solution in the wells was discarded and washed thrice with PBST as above. Subsequently, 100 µl of substrate solution (5-aminosalicylic acid at 1mg/ml concentration in 0.02M phosphate buffer, pH 6.8 and 100 µl of hydrogen peroxidase solution per 10 ml of substrate buffer) was added to each well and allowed to develop the colour in positive wells. The reaction was stopped by adding 50 µl of 3N NaOH to each well.

The results were also confirmed by using Isostrip^{TM} mouse monoclonal antibody Iso-typing kit from Santa Cruz Biotechnology, Inc. Briefly, 150 µl of the culture supernatant containing the test MAb was added to the development tube containing the latex beads bearing anti-mouse kappa (κ) and anti-mouse lambda (λ) antibodies and vortexed gently to suspend the beads completely in the supernatant. The suspension was allowed to stand for 30 sec and an isostrip was placed into the development tube such that the black end which contained the wick was at the bottom. The Isotyping strip provided in the kit contained immobilized bands of goat anti-mouse antibodies corresponding to each of the common mouse antibody heavy chain Isotype (IgG_1, IgG_{2a}, $IgG_{2b,}$ IgG_3, IgM and IgA) and light chain Isotype (κ and λ). Both sides of the strip also contained a positive control band, which indicated that the antibody-coated beads have travelled up the strip. Within 5-10min, blue bands appeared, indicating the isotype of the heavy chain as well as the light chain of the MAb. The reaction was stopped by removing the strip from the development tube once the positive bands appeared. The black portion of the strip was cut to avoid further development of the bands which might otherwise lead to false positive results and the strips were documented by scanning over a conventional scanner.

3.18. Cross Reactivity of MAbs with Other Fungi and Different Strains of *C. albicans*

Cell wall from different isolates of *C. albicans* and other species of fungi was isolated and stored at -80^{o}C. The proteins from the cell wall of the fungi were extracted by boiling in extraction buffer as mentioned above and quantified

using 2D Quant kit. SDS PAGE and electro-transfer was performed. After Ponceau S staining and documentation of band profile, the NCP was immuno-probed against MAbs as described before.

3.19. Cross Reactivity of MAbs with Bacteria

Different bacterial strains (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae*, *Bacillus cereus)* were used to check the cross reactivity of MAbs against them. Bacterial cultures were grown overnight in nutrient broth medium (Beckton Dickinson, USA) and harvested by centrifugation at 6000 rpm for 5min. The bacterial pellet were washed once with PBS, resuspended in 500 µl of SDS sample loading buffer and kept in boiling water for 10min. The contents were then centrifuged at 10000 rpm for 10min at 4^{o}C, the supernatant transferred to fresh labelled tubes and protein quantity was estimated using 2D Quant kit. Equal amount of protein was loaded in each well keeping *C. albicans* cell wall protein as positive control. SDS PAGE and electro-transfer was performed as described before and Western blotting was done using MAbs.

3.20. Monoclonal Antibody Production by Ascites Growth

Monoclonal antibodies were produced in bulk by developing ascites in BALB/c mice. Five BALB/c mice were primed with Freund's incomplete adjuvant (0.5 ml each) intraperitoneally a week before implanting hybridoma cells. The day before implanting hybridoma, cells were split 1:1 in fresh medium (RPMI 1640 with 10% FBS). Next day the cells were pooled after gentle tapping and checked for viability using trypan blue method on a hemacytometer as described above. The pooled hybridoma cells were centrifuged at 1000 rpm for 5min at room temperature and the pellet was resuspended in sterilized PBS to a final concentration of $2x10^{6}$ cells/ml. The primed mice were implanted intraperitoneally with 0.5 ml of the hybridoma cell suspension. The mice were observed daily for the formation of ascites. The formed ascites were tapped after approximately 10 days post-inoculation using an 18-gauge needle followed by two more tapping on alternate days. The ascites fluid was pooled and centrifuged at 1200 rpm for 5min at RT to remove the cells. The

supernatant was further clarified to remove cell debris by filtration through 0.22 µm filter and stored at -20^{o}C until further use.

3.21. Purification of Monoclonal Antibody

Monoclonal antibodies were purified using a pre-packed HiTrap Protein G HP column (GE healthcare) as per the manufacturer's instructions either from culture supernatant or from ascites fluid as follows. All the buffers were prepared in milliQ water and filtered through 0.22µm filter prior to use. The protein G column was connected to the peristaltic pump at the inlet (top end) avoiding air bubbles. The snap-off end of the column outlet was removed and 10 bed volumes of binding buffer (20mM sodium phosphate, pH 7.0) was passed through the column at a rate of 1ml/min to equilibrate the column. Before purification of antibody from culture supernatant, the antibody was precipitated using ammonium sulphate as describe above. The precipitated antibody was dissolved in PBS and dialysed overnight against binding buffer (20mM sodium phosphate, pH 7.0). Ascites fluid was diluted six times in binding buffer before use. The antibody samples either dialysed in binding buffer or diluted in binding buffer were passed through the column at a rate of 1 ml/min. The flow through was collected in a fresh tube and the column was washed with 10 bed volumes of binding buffer to remove non-specifically bound protein molecules. This was assessed by mixing the washings with Bradford reagent in 1:1 ratio which gave no colour with the reagent when the column was washed thoroughly. Elution of the bound monoclonal antibody was done by passing 10 column volumes of elution buffer (0.1 M glycine- HCl, pH 2.7).Fractions (1 ml) of eluent were collected in microfuge tubes containing 200 µl of neutralizing buffer (1 M Tris-HCl, pH 9.0) and the purity of the monoclonal antibody in the eluents were checked by SDS PAGE. Fractions containing the monoclonal antibody were pooled and stored at -20^{o}C after estimating the antibody (protein) concentration.

3.22. Inhibition of Attachment of *C. albicans* with Composite Material

The capacity of monoclonal antibodies to inhibit *C. albicans* attachment to composite/plastic surfaces was analysed. For this, exponentially grown culture

of *C. albicans* was harvested and resuspended in chilled DPBS (Dulbecco's phosphate buffered saline) at a density of $2x10^3$ cells/ml. In a 96-well microtitre ELISA plate, 100 µl *C. albicans* culture suspension was mixed with 100 µl of monoclonal antibody solution (100 µg/ml final concentration) using DPBS as control in place of monoclonal antibodies. The plate was then incubated at 28°C for 1h without shaking after that the contents of the plate were discarded, and washed twice with DPBS. The number of cells sticking to the plate was counted under an inverted phase-contrast microscope (Olympus, Japan). The experiment was performed in triplicate and the data represented mean of all three experiments.

3.23. Inhibition of Germination of *Candida* Cells

Using a 96-well microtitre plate, 100 µl of exponentially grown cell suspension ($2x10^3$ cells/ml) of *C. albicans* were incubated with 100 µl of the test MAb solution (1mg/ml) at 37°C for 4h. The plate was observed under phase contrast microscope for the germ tube formation. DPBS was taken as control. The experiment was performed in triplicate and the percentage of germination was determined by the following formula:

Inhibition of germination =Number of germ tube/Total number of cells in the given field) x 100

3.24. Effect of MAbs on Colony Forming Units (cfu)

The effect of monoclonal antibodies on cfu was evaluated by incubating $1x10^3$ cells of *C. albicans* with 100 µl of test antibody solution (0.5mg/ml) in DPBS at 28°C for 16h and plating them onto SDA plates. The reduction in cfu was calculated by comparing it with control that contained an equal amount of DPBS without any antibody.

3.25. MTT Assay

Candidacidal activity of monoclonal antibodies was determined by MTT assay. Briefly, 100 µl of cell suspension ($1x10^4$ cells/ml) was incubated with 100 µl of antibody (1mg/ml) and incubated for 16h at 35°C. After incubation, cells were centrifuged at 1000 rpm for 5min and to the pellet 100 µl of MTT (0.5mg/ml in

RPMI) was added and incubated for 1h. After incubation, the suspension was centrifuged at 5000 rpm for 5min and 100 µl of DMSO was added to the pellet and the absorbance of the dark formazan product formed was read at 550nm. DPBS and amphotericin B were used as negative and positive control respectively. The optical density obtained for AMB was considered as 100 % inhibition and that of DPBS as 0 %. The formula used to calculate percent inhibition was [100 – (x-OD_{AMB}/OD_{DPBS} – OD_{AMB}) x 100], where x is the OD of test antibody.

3.26. Fluorescence Activated Cell Sorting (FACS analysis)

Flow cytometry analysis was performed to determine candidacidal activity. Briefly, 500 µl of exponentially grown *C. albicans* cell suspension ($1x10^5$ cell/ml) was mixed with 500 µl of test monoclonal antibody (1mg/ml) and incubated for 1h at 28^oC. Positive and negative control as mentioned above was used in this experiment. After incubation, 2 µl of propidium iodide (1mg/ml in water) and 10 µl of fluorescene diacetate (5mg/ml in acetone diluted 1:20 in DPBS) was added and stored on ice for not more than 30min before analysis on flow cytometer (Becton and Dickinson, USA). The experiment was repeated three times and the data represented was best of three replicates.

3.27. Phagocytosis Assay

Mouse macrophage cell line J774, actively growing yeast cells and germ tubes was used for phagocytosis experiments. The cell line J774 was sub cultured two days before performing the phagocytic assay. Sub culturing was done by gently dislodging the cells using a cell scrapper (Greiner bio-one, Austria). 20 µl of cells were mixed with trypan blue solution (1:1) and cell counting was done using a hemacytometer. $4x10^6$ cells were seeded in a 250 ml tissue culture flask in DMEM with 10% FBS and incubated at 37^oC in atmosphere of 5% CO_2.

3.27.1. Treatment of Yeast and Germ Tubes with MAb

The exponentially grown yeast cells were harvested by centrifugation at 5000 rpm for 5min, washed twice with sterilized DPBS by centrifugation and

resuspended in DPBS containing test monoclonal antibody (1mg/ml) at 1 x 10^6 cells/ml and incubated overnight (16h at 37^{o}C) with gentle shaking (50 rpm). Germ tube induction was carried out in hyphal induction medium (0.67% YNB, 0.2% NAG and 2% glucose). After 1h, the yeast cells with germ tubes were harvested by centrifugation at 1200 rpm for 5min, washed twice with PBS by centrifugation and resuspended in PBS containing purified MAb (1 mg/ml) at 1 x 10^6 cells/ml and incubated overnight as above with mild shaking (50 rpm). The yeast cells and germ tubes suspended in PBS without MAb served as control.

3.27.2. Phagocytosis and Geimsa Staining

Macrophage cells sub-cultured 48h prior to experiment were dislodged by scrapping using a cell scrapper and seeded into 6-well tissue culture plates at 2 x 10^6 cells per ml (2 ml per well) and allowed to adhere for 6h. Control and monoclonal antibody treated yeast and germ tubes were centrifuged at 5000 rpm, washed thrice with and resuspended at a concentration of 4 x 10^6 cells/ml in incomplete DMEM (without FBS). The cells were pipetted out gently to separate cell aggregates. Media was removed from the 6-well tissue culture plates and macrophage cells were washed with incomplete DMEM to remove the traces of FBS. The yeast and germ tube cell suspensions were added to macrophages in a ratio of 1:1 and kept at 37^{o}C in an atmosphere of 5% CO_2 for 2h. After 2h incomplete DMEM was replaced by DMEM with 10% FBS containing antimycotic and antibiotic solution and phagocytosis was allowed for one more hour. After 1h, the media was removed from the wells and the cells were washed thrice with incomplete DMEM to remove non-phagocytosed yeasts/germ tubes adhering to the surface of the well and the macrophages and washed subsequently twice with DPBS. The cells were fixed with 100% methanol (1 ml/well) for 30 seconds at RT. Geimsa stain (diluted 1:10 with PBS) was added (1 ml/slide) to each well and allowed to stand for 30min at 37^{o}C. The excess stain was removed and the wells were washed thrice with PBS (1min each). The stained cells were observed under a phase contrast

microscope (Olympus, Japan) and photographed using a high resolution camera (Nikon, Japan).

3.28. Epitope Localization on the Surface of *C. albicans*

Localization of the epitope recognized by MAb on intact *C. albicans* cells (yeast cells and hyphae) was performed by indirect immuno-fluorescence microscopy. The exponentially growing yeast cells and hyphae of *C. albicans* were collected by centrifugation, washed twice with PBS and suspended in the same at a cell density of $1x10^3$ cells/ ml. The cell suspension was placed on a cover slip (10 µl/cover slip) coated with poly-L-lysine (Sigma) and fixed with 4% para-formaldehyde. The cover slips were blocked with 1% BSA in PBS in a moist chamber at 37^oC for 1h and washed twice (2min each) with PBS under mild shaking. To each blocked cover slip 15 µl of MAb (50 µg/ml in PBS) was overlaid and incubated in a moist chamber for 1h. The cover slips were washed with four changes of PBS as mentioned above and 15 µl of FITC-conjugated anti-mouse antibody (FITC –conjugated antimouse IgG, IgM, or IgA) at a dilution of 1:100 in PBS was overlaid on it and incubated for another hour. The cover slips were washed finally with four changes of PBS and mounted in mounting medium containing antifade by inverting them over a clean glass slide. The margins of the cover slips were sealed with nail enamel and the slides were observed under a phase contrast fluorescence microscope and documented.

3.29. Protection against Systemic Challenge by Passive Transfer of Monoclonal Antibodies

A group of five female BALB/c mice were taken for each monoclonal antibody to be tested. These mice received 200 µg of test antibody (1mg/ml) intraperitoneally 4h before challenge (with *C. albicans*) and six successive 100 µg doses at 1, 2, 3, 4, 6 and 9 days post challenge. The mice were challenged with 200 µl (2 x 10^5 cells) of cell suspension of *C. albicans* in normal saline intravenously via lateral tail vein. One irrelevant MAb was also taken in experiment as control. Protection was evaluated by monitoring animal survival for 15 days. After day fifteen, the mice were sacrificed and both kidneys were

removed and weighed. The kidneys were homogenized in 5 ml of sterile PBS in a tissue homogenizer, and cfu/gram of kidney tissue were determined by usual dilution and plating technique.

3.30. Assessment of *in vivo* Diagnostic Potential of Monoclonal Antibody

3.30.1. Biotin Labelling of Monoclonal Antibody

Purified monoclonal antibody was biotinylated using biotinylation kit from GENEI as per manufacturer's instructions. Briefly, 100 µl of conjugation buffer was added to 1 ml of monoclonal antibody solution (1mg/ml) in PBS. The contents were then transferred to a vial containing 0.5mg BAH-Sulfo-NHS (Biotinamidohexanonate-N-hydroxy-sulfosuccinimide ester and incubated for 2h at 4^{o}C with mild stirring. The mixture was loaded on streptavidin column to allow the biotinylated monoclonal antibodies to bind to the column. The flow through was collected in a fresh tube and stored at -20^{o}C. The column was washed with 10 bed volumes of PBS and eluted with 8M guanidine-HCl, pH 2.7. Monoclonal antibody (biotinylated) presence was checked using Bradford assay. The biotinylated monoclonal antibody was used to check its diagnostic potential by detecting the antigens present in the serum of the infected mice.

3.30.2. Diagnosis Potential of Monoclonal Antibody in Mouse Model of *C. albicans* Infection

Six BALB/c mice were challenged intravenously with 200 µl (2 x 10^{5} cells) of yeast suspension of *C. albicans*. Uninfected BALB/c mice served as negative control. After every three days blood was drawn through the tail vein to check the presence of antigens recognized by the monoclonal antibody that might have been expressed during the course of infection from both infected as well as control mice. Serum (50 µl) from infected as well as uninfected mice was mixed with 150 µl of PBS and stored at -20 C until used further. The microtitre ELISA plates were coated with 100 µl of the stored serum (in PBS) of infected as well as control mice and kept overnight at 4^{o}C. Wells coated with 100 µl (100 µg) of hyphal GPI CWP served as positive control. The coated plates were washed and blocked as mentioned before with 150 µl of BSA (1%). The biotinylated monoclonal antibody (1:1000) was used to probe the presence of

Candida antigen in the serum of the infected mice. The plates were then incubated with 100 µl of biotinylated monoclonal antibody for 1h at 35^{o}C. Later the plates were washed thrice with PBS-T20 (5min each) and incubated with 100 µl of horse radish peroxidise (HRP) enzyme conjugated streptavidin polymer (sigma) diluted 1:200 at 35^{o}C for 45min. The plates were again washed thrice with PBS-T20 (5min each) and developed with OPD as mentioned earlier.

3.31. Detection of Antigens Recognized by MAb in *Candida* Positive Patient Sera Using ELISA

The monoclonal antibody was checked for its ability to detect antigens of *C. albicans* in serum of the persons who have been confirmed for candidiasis in our laboratory by culture method as well as *Candida* specific antibody detection method. Fifteen such patient sera and nine sera from healthy donors were screened for presence of the antigens by ELISA method essentially as described before with minor changes. Briefly, patient sera (18 samples) were diluted in coating buffer (1:100) and 100 µl of diluted serum was added to each well of micro titre plates in duplicates and incubated overnight at 4^{o}C. Hyphal cell wall proteins (100 µg) served as positive control. The plate was washed with PBS-T20 once for 5min and the coated wells were blocked by adding 150µl of 1% (w/v) bovine-serum albumin in PBS for 90min at 37^{o}C. Blocking solution was removed and the wells were washed once with PBS-T20 (5min). Purified MAb in PBS (100 µl, 1mg/ml) was added to each well and incubated for 90min at 37^{o}C. Plate was washed thrice with PBS-T20 (5min each) and incubated with 100 µl of peroxidase-conjugated anti-mouse IgG, diluted 1:5000 in PBS-T20 for 90min at 37^{o}C. Tetramethyl benzidine (TMB) substrate solution (RnD systems, USA) was prepared by mixing solution A and solution B in equal volumes just before use and 100 µl was added to each well and incubated in dark at RT for 30min. The reaction was stopped with 10 µl of 7% H_2SO_4 and optical densities were read on a micro titre plate reader (Molecular Devices, USA) at 450 nm.

3.32. RNA Isolation from Hybridoma Cells

Total RNA was extracted from hybridoma cells using RNeasy MiniKit (Qiagen, Germany) according to manufacturer's protocol as describe above. The extracted RNA was checked for its quality on formaldehyde agarose gel (1.5%) prepared in 1xMOPS buffer containing 2.2M formaldehyde. Two microlitres of extracted RNA was mixed with 10 µl formamide, 4 µl formaldehyde, and 2 µl of 1mg/ml Etbr and heated at 85^0C for 15min and loaded onto the gel with 2 µl of loading dye. Before proceeding for RT-PCR, the RNA was treated with DNase for complete removal of DNA. The RNA (20 µl) sample was mixed with 1xDNase buffer and 10U of DNase and incubated at 37^oC for 15min followed by addition of 10 µl stop solution. These samples were then heated at 85^oC for denaturation of enzyme and RNA.

3.33. Reverse Transcriptase PCR

Reverse transcription was carried out with 2 µg of total amount of RNA. The Qiagen one step RT-PCR kit and IgG1 specific primer was used.

Primer used for light chain

MKV1= ATGAAGATTGCCTGTTAGGCTGTTGGTGCT

MKV2= ATGTGGGGACT(CT)TT(GT)T(CT)C(AC)TTTTTCAATG

MKV3= ATGTATATATGTTTGTCTATTTCT

MKV4= ATGGAAGCCCCCAGCTCAGCTTCTCTTCC

MKC = ACTGGATGGGAAGATGG

Primer used for heavy chain

MHV1 =ATGAAATGGAGCTGGGGCAT(GC)TTCTTC

MHV2 =ATGAGAGTGCTGATTCTTTTGTG

MHV3=ATGGGCAGACTTACCATTCTCATTCCT

MHCG1=CAGTGGATAGACAGATGGGGG

MHCG2=CAAGGGATAGACAGATGGGGC

RT-PCR reactions for IgG1 were performed using Qiagen one step RT-PCR kit as explained above. The reaction involved reverse transcription at 50^{o}C for 30min, initial denaturation at 95^{o}C for 10min, followed by 35 cycles in series of denaturation at 95°C for 30s, annealing at 60^{o}C for 45s and extension at 72°C for 90s, with a final step of one cycle at 72°C for 10min to final extension and the final products were checked on 1% agarose gel electrophoresis.

3.34. Development of Biologically Active Antibody Derived Peptides

3.34.1. RT PCR Product Sequencing

RT-PCR products were sequenced with an ABI Prism automated DNA sequencer (model 3100, version 3.0: Applied Biosystems, Warrington, United Kingdom) at DNA sequencing facility, South Campus, Delhi University. Successfully obtained clean sequences were matched with available database BLASTn (www.ncbi.nlm.nih.gov/BLASTn). Maximum matched sequences were aligned with obtained sequence through CLUSTALW, a database for alignment of DNA and protein sequences (www.ebi.ac.uk/clustalw)

3.34.2. Peptide Designing

The nucleotide sequence obtained was subjected to *in silico* analysis in order to obtain protein sequences through BLASTx (NCBI). The best HITS obtained from BLASTx results were subjected to a software program" Predicted Antigenic Peptides" (http://bio.dfci.harvard.edu/Tools/index.html) to identify complementarity determining regions (CDRs) regions. Amino acids adjacent to the CDRs were also considered in order to design the sequence of dodecapeptides for synthesis.

3.34.3. Peptide Synthesis

For the synthesis of peptides, Resin-Rink Amide AM resin (RAM) (1% divinyl benzene, 100-200 mesh, 0.63 mmol/g substitution), anhydrous solvents were used for the reactions. Initially, F-moc group from RAM resin was removed using 30% (v/v) piperidine in DMF for 5min and 25min followed by washing of the resin with DMF (3×2 ml), methanol (3×2 ml), DCM (3×2 ml), and ether (3×2 ml). The C-terminal Fmoc-amino acid was then treated with resin and the

reaction mixture was stirred at room temperature for 6h and subsequently washed as mentioned above. Deprotection of F-moc was repeated as above and further confirmed by the Kaiser test. Calculated amount of amino acid, HOBt and DIC in DMF were transferred into vial and this mixture was stirred for 5min and loaded on the syringe and total volume was made up to 1.5 ml and stirred on orbital shaker for 6h at room temperature. After 6h stirring solvent was drained out from the reaction mixture and washed as mentioned above, again the loading of amino acid was confirmed by Kaiser Test. The cleavage of the peptide from the solid support was achieved by treating the peptide resin with reagent K (82.5% TFA / 5% phenol / 5% thio-anisole / 2.5% 1, 2 ethandithiol / 5% water) at room temperature for 2h. Cleavage mixture was collected by filtration and the product was precipitated using anhydrous ether. The white precipitate so obtained was centrifuged, washed with ether and dried. Finally the white residue was dissolved in t-butanol/water (4:1) and lyophilized to get the peptides. This was characterized by using ESI-MS. The mass distribution observed in ES-MS spectrum was compared with the calculated mass distribution pattern using software.

3.34.4. *In vitro* Antifungal Activity of Paratope Derived Peptides

The synthesized peptides were tested for their *in vitro* antifungal activity as per guide lines of CLSI against *C. albicans.* Initially 4 mg/ml solutions of peptides were made in 5% DMSO. To each well of a sterile 96-well flat bottom microtitre plate, 150 μl RPMI 1640 buffered with MOPS was added except the fourth column where 270 μl broth was used. To this fourth column 20 μl of the test peptide was added and diluted two-fold from 4^{th} through 11^{th} column. Appropriate growth control, test compound control and blank were also set. Finally 20 μl of the freshly prepared inoculum of *C. albicans* was added to each well (final cfu $1x10^3$/ml). To the first row 5 μl of 4% formaldehyde was added to serve as blank. The plates were incubated at 35^oC in a moist chamber for 24 - 48h. The optical density was recorded on a microtitre plate reader at 492 nm to determine the minimal inhibitory concentrations.

Results

4.1. Acquired Amphotericin B Resistance in *Candida albicans*

Generating resistant strain via continuous exposure to drug can be considered as selection for the evolution of drug resistance. *C. albicans* (ATCC 10231) was serially passaged under the influence of increasing concentration of amphotericin B on the basis of initial MIC (0.06 µg/ml). After ~60 passages the MIC was found to be 128 µg/ml and the resistant strain was termed as AMB-R. In this study, serial passaging of *C. albicans* in AMB containing media may have induced strong selective pressure to acquire the resistance. After ~60 generation of exposure to amphotericin B, AMB-R was grown in SDB without amphotericin B to access the stability of resistance. MIC was determined on regular interval up to 60 days. There was no change in MIC which indicates stability of the acquired resistance. Amphotericin B treatment of parent strain infected mice resulted in 100% reduction in cfu from kidney tissue whereas in case of AMB-R strain infected mice it did not reduce the cfu significantly (Table 1) thereby indicating an acquired, enhanced and sustainable resistance in the newly developed AMB-R strain.

Table 1: Comparison of cfu counts of *C. albicans* (parent and AMB-R strains) in kidney tissue of Swiss mice

Strain used	Mice/group	Treatment	Log10 cfu / g of kidney± SEM
C. albicans	6	Control	5.396±0.149
C. albicans	6	AMB	Nil
AMB-R	6	Control	5.645 ± 0.172
AMB-R	6	AMB	4.692 ±0.134

Note: $4x10^5$ cells of *C. albicans* (parent or AMR-R strain) were given intravenously in Swiss mice. The mice were treated with amphotericin B (5mg/kg p.o.) for 7 days and cfu were determined in kidney tissue on day 9.

4.2 Time Kill Assay

Resistance against fungicidal antifungal agents can be assessed by calculating the reduction in cfu at different time intervals. For time kill assay the concentration of amphotericin B used against the parent strain was 0.06 µg/ml (MIC) whereas for the AMB-R strain it was 128 µg/ml (MIC). There was a sharp decrease ($\geq 2\log_{10}$) in cfu *in vitro* after 4 hour of incubation of parent strain of *C. albicans* ATCC10231 at 2x MIC which continued to decrease till

16h after which no viable cells could be detected while at MIC level a gradual decrease in cfu by ≥1log_{10} was observed indicating its susceptibility to AMB. There were no major changes in cfu at ½ MIC up to 24 hours, however it increased slightly thereafter. Whereas in case of AMB-R strain there was 1.5log_{10} reduction in cfu at 2x MIC but it did not follow the killing pattern observed for parent strain (Figure 1).

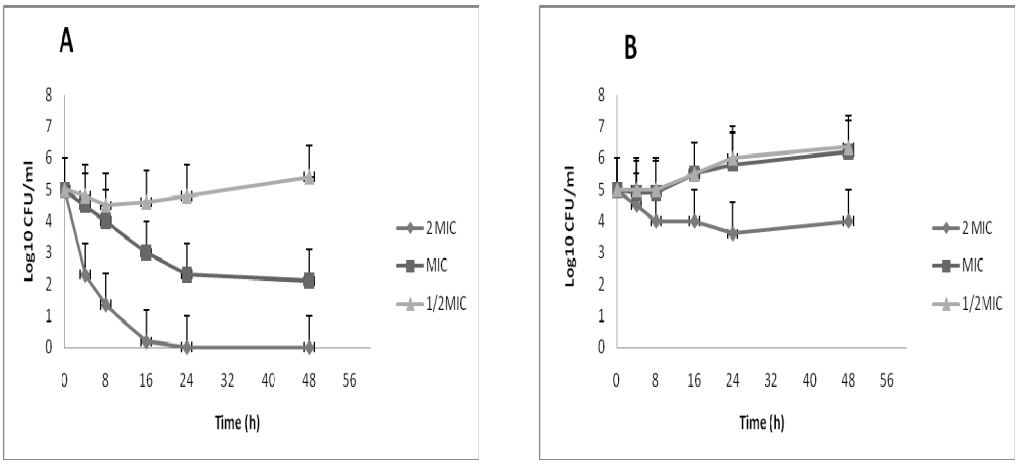


Figure 1. Time kill assay for amphotericin B against *C. albicans* parent and AMB-R strains. The data represented is the mean±SD of three experiments. (A) Time Kill curve for parent strain of *C. albicans*, MIC used 0.06 µg/ml (B) Time Kill curve for AMB-R. MIC used 125 µg/ml.

4.3. Morphological Characterization

Both the strains were grown in hyphal induction medium and subjected to fluorescence microscopy using Calcofluor white. The results are shown in Figure 2. The parent strain of *C. albicans* ATCC10231 exhibited true hyphae morphology where as in AMB-R strain morphology was changed to pseudohyphae.

4.4. Germ Tube Formation Assay

The germ tube formation capacity of both parent and AMB-R strains of *C. albicans* was compared at different concentrations of AMB. The germ tube formation was found to be dose dependent in the parent strain as it increased gradually with the reduction in drug concentration. The germ tube formation in

parent strain increased suddenly at lower concentration (3.9 µg and below) of amphotericin B whereas in case of AMB-R strain there was a gradual increase with a significant difference at lower concentration as compared to parent strain. The dose response to amphotericin B of parent as well as AMB-R strain is illustrated in Figure 3.

4.5. Dot Blot Assay for β-1,6-glucan synthase Activity

A dot blot protocol was used to detect the β-1, 6-glucan synthase activity in both parent as well as the AMB-R strains of *C. albicans.* Aliquots from the reaction mixture were spotted onto a nitrocellulose membrane and allowed to dry before probing the spots with polyclonal antibodies. The β-1,6-glucan signal consistently increased over time (up to 4h).The availability of mutants with altered cell wall levels of β-1,6-glucan offers an alternative way to examine the specificity of the *in vitro* assay. Here, the results indicated that the cell wall of AMB-R contained altered levels of cell wall β-1,6-glucan as compared to its parent strain of *C. albicans* as shown in Figure 4. The dot blot was scanned and densitometry was performed using adobe photoshop software. The relative intensity of the spots was calculated and plotted (Figure 5). The relative intensity data indicated that there was no significant difference in glucan synthase activity in yeast membranes of both parent as well as AMB-R for 0 and 1h after which, the synthesis of glucan synthase started increasing significantly ($P<0.001$) in membranes of parent *C. albicans* than AMB-R. On the other hand, the glucan synthase activity was significantly higher ($P<0.001$) in hyphal form of parent *C. albicans* than the pseudohyphal form of AMB-R from 0h onwards. The basal level of glucan synthase activity in hyphae of *C. albicans* was 6.25% higher than AMB-R at 0h, which further increased to 15.26%, 20% and 36% at 1h, 2h and 4h respectively.

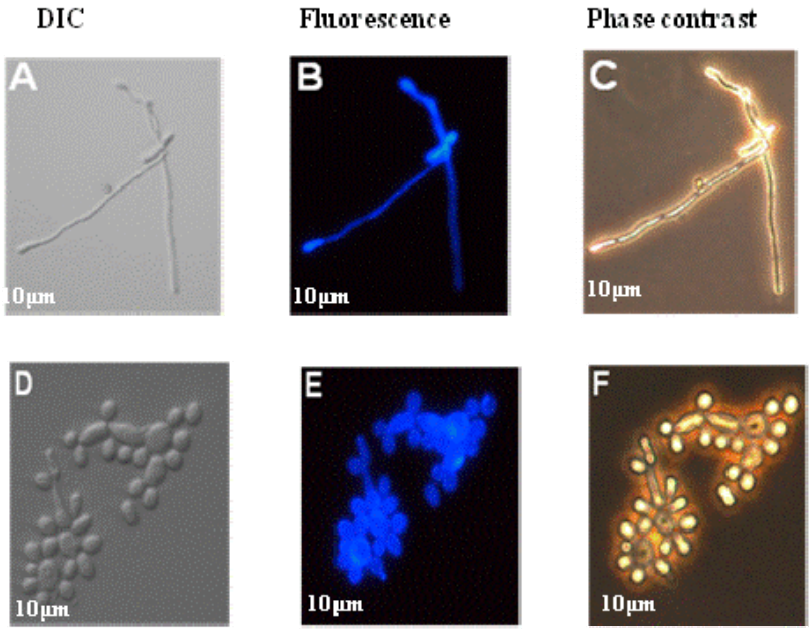


Figure 2: Morphological differences in *C. albicans* parent strain and AMB-R strain in YNB +10%FBS. A,B,C represent typical hyphae formation in *Candida albicans* ATCC10231, parent strain where as D,E,F show pseudohyphae formation in *C. albicans* AMB-R strain.

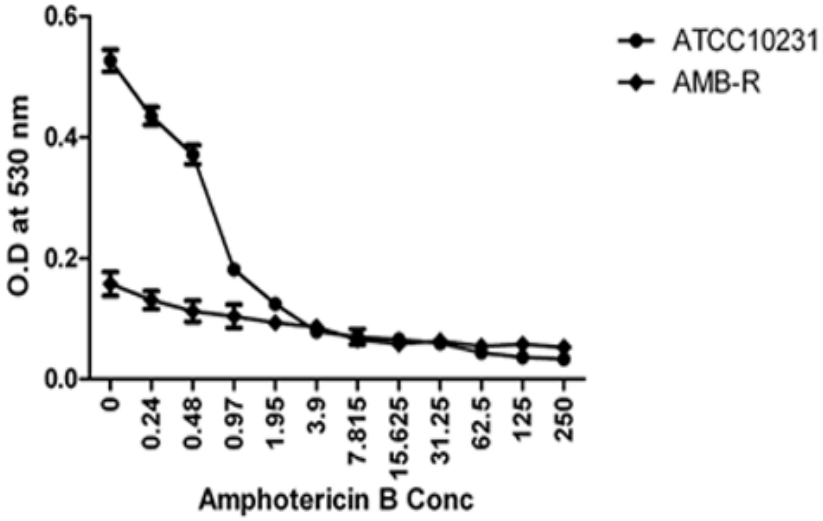


Figure 3. Comparative dose response curve for germ tube formation in AMB-R strain and *C. albicans* (ATCC10231) parent strain. Both the strains were subjected to various AMB concentrations (250 µg/ml and below). AMB-R strain showed significant reduction in germ tube formation as compared to the parent strain (*C. albicans* ATCC10231)up to drug concentration of 0.97 µg.($P<0.001$).The data presented is the mean of three experiments.

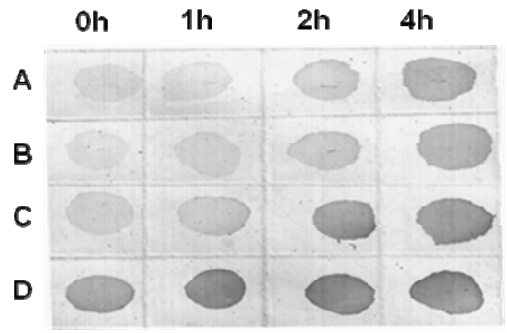


Figure 4. β-1,6-glucan synthase activity of membranes prepared from yeast and hyphal/pseudohyphal cells of parent *C. albicans* and AMB-R. The reaction was allowed to run for 4h and the reaction mixture was blotted on nitrocellulose membrane, probed with polyclonal antibodies developed against *β*-D-glucan cell wall. A and B represents glucan synthase activity in membranes of yeast and pseudohyphae form of AMB-R. C and D represent glucan synthase activity in yeast and hyphal form of parent *C. albicans*

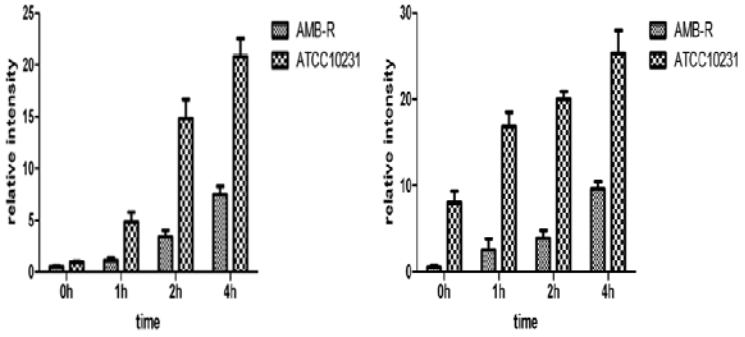


Figure 5. The relative intensity of the dot blot signals. A. relative intensity signals for yeast form. B. Relative intensity for hyphal/ pseudohyphal form

4.6. Spectrophotometric Semi-Microdetermination of Ergosterol

The sterol quantitation method (SQM) involves the quantitation of membrane sterols of fungal cells. The total ergosterol content was determined for both parent and AMB-R strains. Mean ergosterol content was calculated as percentage of the wet weight of the cell. A significant decrease of 56.6% of total ergosterol content in AMB-R strain as compared to parent strain was observed (Figure 6).

4.7. Extracellular Proteinase Activity

In the present study the Sap activity was measured spectrophotometrically using BSA as substrate (Figure 7). The Sap activity (Units/cfu/ml) in AMB-R strain was 0.7010 on day 1 and 1.669 on day 6 while the activity in parent strain was found to be 0.25 on day 1 that increased up to 0.7173 on day 6. The difference between Sap activities in both the strains was statically significant at all the time points ($p<0.001$). Our results indicated enhanced activity of secreted aspartyl proteinase in AMB resistant strain AMB-R than parent *C. albicans* strain.

4.8. Extracellular Phospholipase Activity

Both parent and AMB-R strains were screened for extracellular phospholipase activity. The phospholipase activity of the isolate was interpreted positive when a precipitation zone was visible around the growth. Phospholipase activity was measured by dividing colony diameter by the diameter of the precipitation zone (Pz) around the colony formed on the plate. A Pz (in mm) of 1.0 was evaluated as negative (–), 0.99–0.9 as weak (+), 0.89–0.8 as mild (++), 0.79–0.7 as relatively strong (+++) and 0.69-below (++++) as very strong positive. The Pz (in mm) for AMB-R was 0.382±0.038 that showed very strong presence of phospholipase activity as compared to its parent strain 0.664±0.047 which was relatively strong (Figure 8). The enhanced activity of extracellular phospholipase can be indication of increased resistant of *C. albicans* towards AMB.

4.9. Expression Analysis of *ERG11*

The expression level of *ERG11* was analysed using Reverse Transcription PCR. After amplification the DNA bands were documented and analysed using Adobe Photoshop densitometry. The relative intensity of DNA bands was calculated and presented in graph (Figure 9 A and B). The expression level of *ERG11* in yeast as well as pseudohyphae of AMB-R was compared with that in yeast and hyphae of parent strain. The results indicated that the relative intensity of AMB-R was 2.345±0.624 as compared to parent *C. albicans* was 10.312± 1.68 which indicated reduction of expression level to

22% in case of AMB-R. Likewise, the relative intensity of hyphae (in parent strain) and pseudohyphae (in AMB-R) was 15.961±1.44 and 2.083±0.375 which indicated the reduction in expression level of *ERG11* in AMB-R was 13.33%.

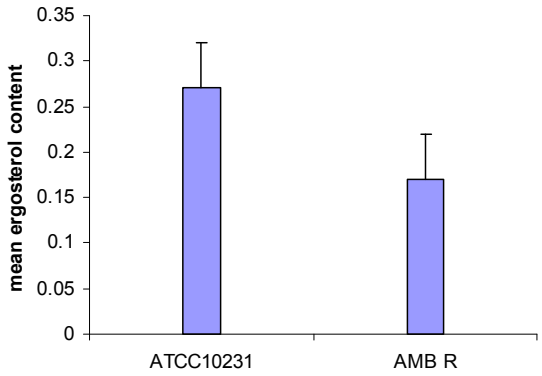


Figure 6. Comparison of ergosterol content in C. albicans AMB-R and *C. albicans* (ATCC10231) parent strain. Stationary phase grown cells were subjected to total ergosterol content extraction. The bar diagram represented the mean ± SD of three repetitions.

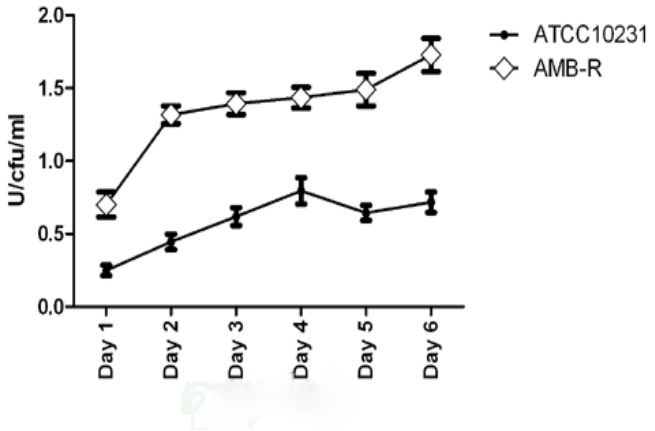


Figure 7. Comparison of SAP activity (units/cfu/ml) in *C. albicans* AMB-R and *C. albicans* (ATCC10231) parent strain. SAP activity was determined on three different occasions using BSA as substrate. AMB-R exhibited significantly (P<0.001) higher enzymatic activity as compared to ATCC10231.

4.10. DNA Isolation

The genomic DNA was isolated from *C. albicans* and its resistant strain AMB-R using two methods. The first one was based on conventional phenol: chloroform method after lysis and the second on the commercial silica based DNA binding columns. The isolation of DNA samples using DNA binding columns (DNeasy plant mini kit, Qiagen) took less time and it was of good quality as compared to that isolated by conventional method. The ratio at 260/280nm and the concentration of DNA was measured on spectrophotometer (UV/VIS 911A, GBC Scientific Equipments, Australia) for all the DNA samples**.** The ratio at 260/280nm with both the methods for all the strains ranged between 1.7-1.8, whereas the concentrations of DNA from ATCC 10231 yeast and hyphal DNA by phenol: chloroform method were 760, 747 ng/ml respectively, whereas the DNA concentration for AMB-R yeast and pseudohyphae were 620, 685 ng/ml while it was little higher (1050, 940, 840, 930 ng/ml) with DNeasy plant kit with less smearing. Thus for further studies the DNA was isolated by DNeasy plant mini kit.

4.11. PCR Amplification, Sequencing and CLUSTALW Analysis

Two genes *ERG11* and *CSP37* involved in antifungal drug resistance mechanisms were amplified (PCR amplification) using gene specific primers. The amplification conditions were same except for annealing temperature. The product size for *ERG11* and *CSP37* were 1.5 kb and 950bp. The products were purified and sequenced using single primer. After sequencing, BLASTn was used to identify the DNA sequence. After confirming the sequence of both *ERG11* and *CSP37*, both the sequences were subjected to CLUSTALW software for pair wise alignment in order to identify the nucleotide changes at gene level for both the strains (Figure 10).

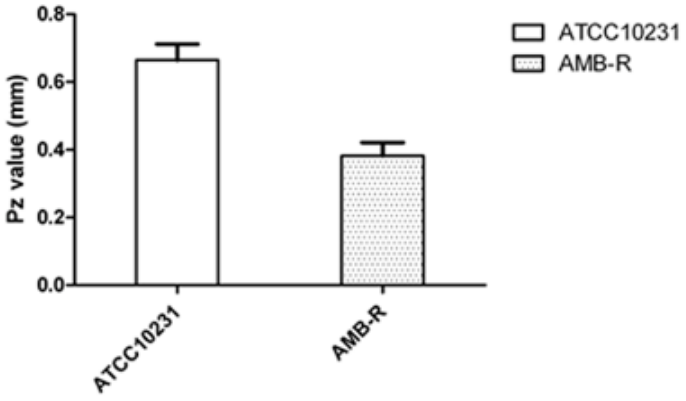


Figure 8. The precipitation zone (Pz) in mm showing phospholipase activity in parent *C. albicans* and AMB-R strains. The Pz value represents ratio of colony diameter and the diameter of the precipitation zone.

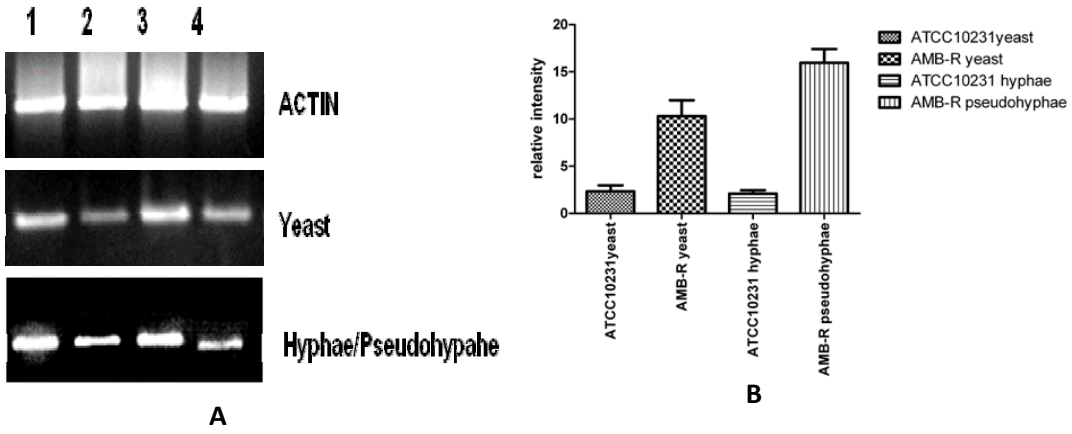


Figure 9. (A) The expression analysis of *ERG11* on the gel. The expression level was compared with the housekeeping gene actin. (B)The expression level in yeast and hyphae was compared in both parent *C. albicans* and AMB-R and the relative intensity was calculated ($P<0.001$).

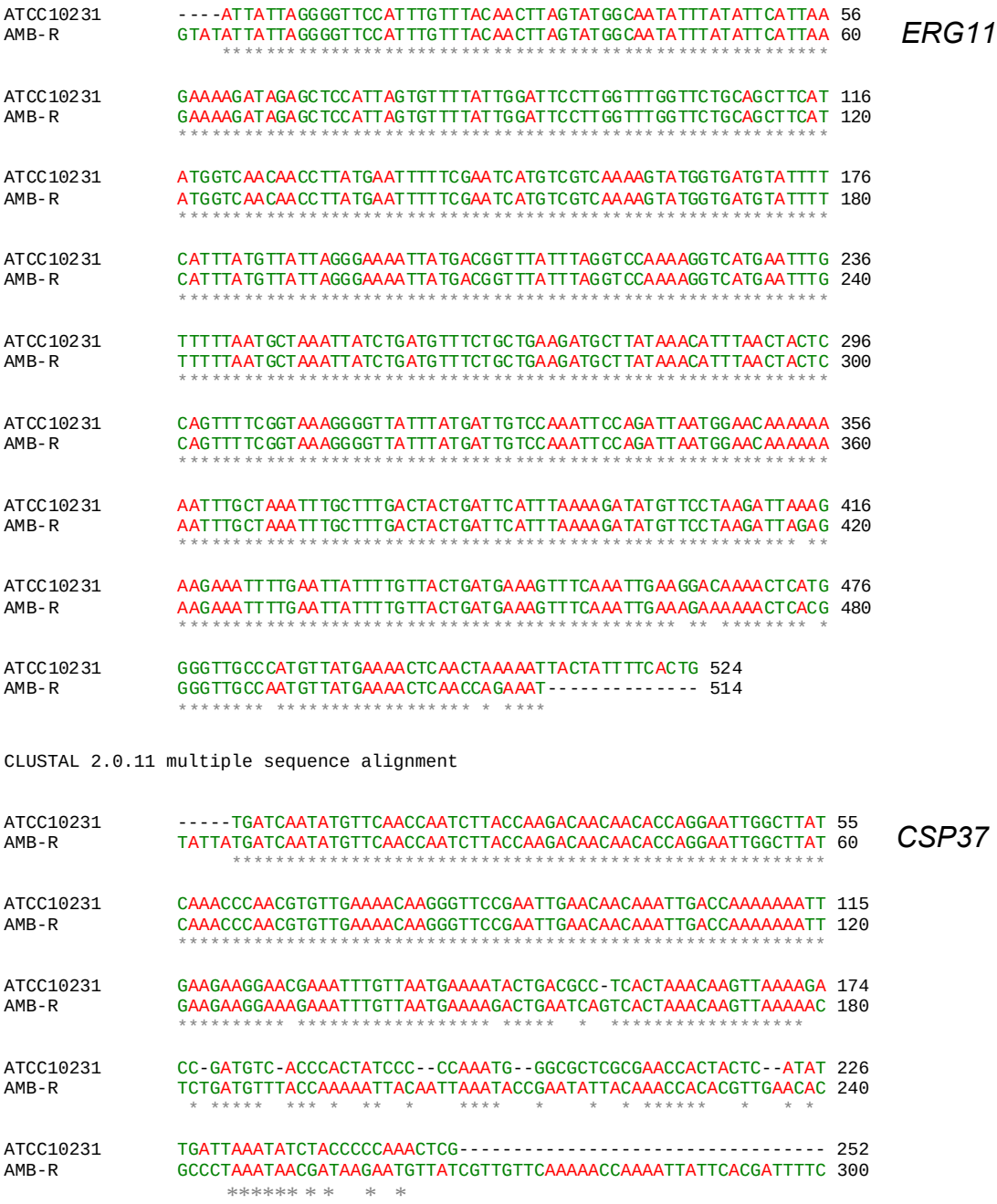


Figure 10. Pair wise nucleotide sequence alignment for parent *C. albicans* and AMB-R of ERG11 and *CSP37* using CLUSTAL W software.

In *ERG11* The mutation were observed at 462G - 466A, 464C-468A, 475T-479C, 485C-489A,503T-507C,505A-509G in parent *C. albicans* ATCC10231-AMB-R respectively.

In *CSP37*,The mutation were observed at 125C-130A, 154T-149G, 135C-154A, 136G-155T, 138C-157A, in parent *C. albicans* ATCC10231-AMB-R respectively.

4.12. Exposure to H_2O_2 and Menadione

Amphotericin B resistant Strain and its parent strain were exposed to oxidative stress inducing agent menadione and H_2O_2. The enzymes Catalase (CAT),Glutathione peroxidise (GPx) and Glutathione S Transferase (GST) activity were determined .The three antioxidative enzymes were found to be significantly higher in AMB-R under normal and stress conditions as compare to its parent (Figure 11 and 12).

There was no reduction in the cfu of the yeast cells under H_2O_2 and menadione stress at different concentration, which was confirmed by dilution and plating method to ensure the homogeneity in live cell population for both the strains. The concentration of oxidative stress agents used for the enzymatic activity in hyphal/pseudohyphal forms were the same as used in case of yeast forms of both the strains.

As the drug resistance is resultant of continuous stress under drug pressure, the basal level of the antioxidative enzymes were also estimated in the experiments. The basal level of catalase in yeast form of parent strain was 86.29 ±4.64 where as it was found to be 166.48±7.96 in AMB-R. Likewise, the GPx and GST activity were also higher in AMB-R as compared to its parent (49.20±9.97, 83.75±22.99, 90.77±18.42 and 169.19±20.86). Similarly for hyphal/pseudohyphal forms, the basal levels of anti-oxidative enzymes were higher in AMB-R than its parent respectively (CAT-69.040±7.32 134.063±15.93, GPx -46.44±6.65 and 129.72±22.34, GST-79.65±12.74 and 116.80±23.4).

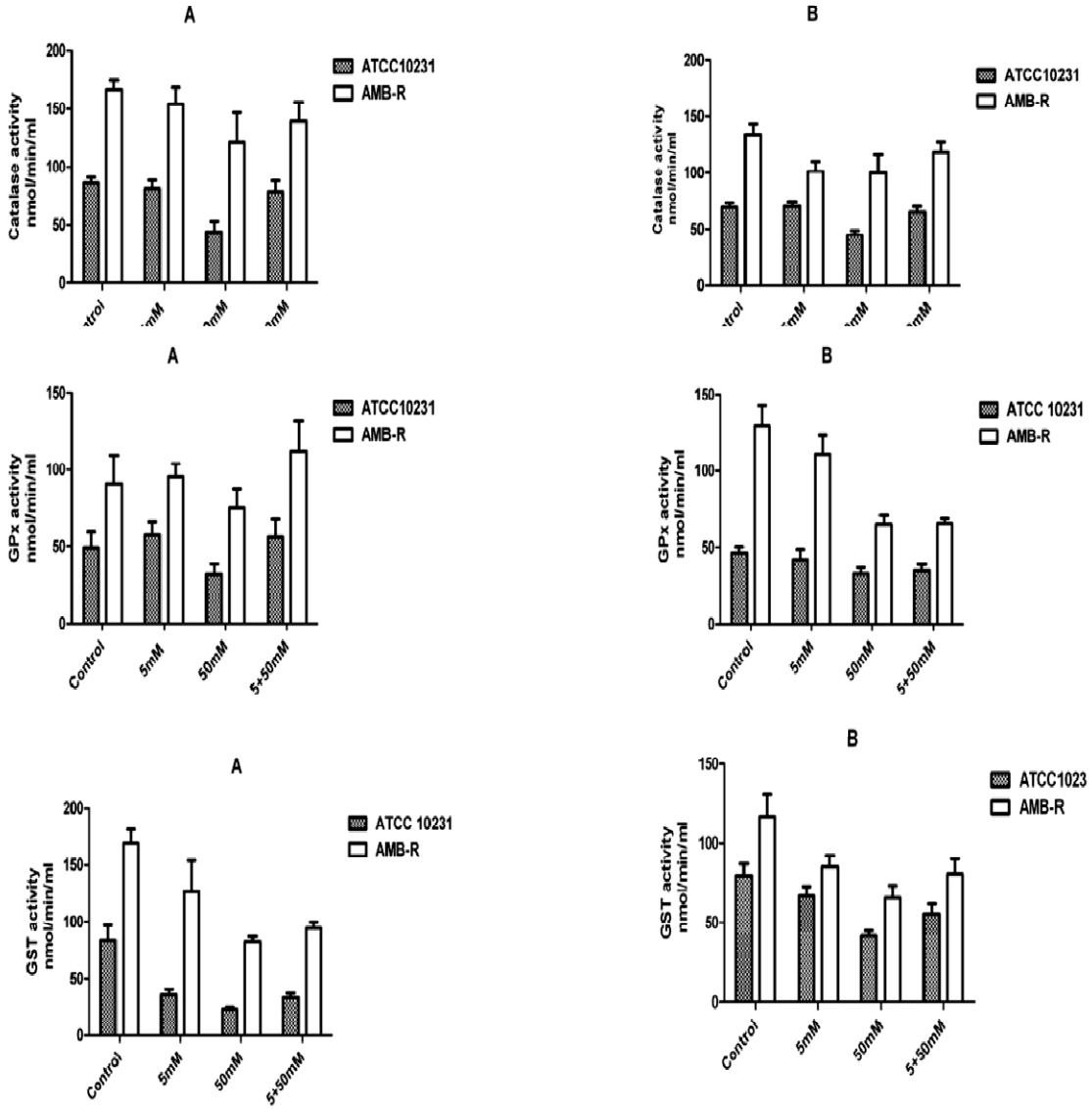


Figure 11. Yeast and hyphal/pseudohyphal form of both parent *C. albicans* and Resistant AMB-R morphological form were challenged to oxidative stress at 5mM, 50mM and 5+50mM H_2O_2.The activities of antioxidative enzymes catalase, glutathione peroxidase and glutathione S tranferase were estimated in the cell lysates. The differences between the enzymatic activities are statically significant ($P<0.001$).

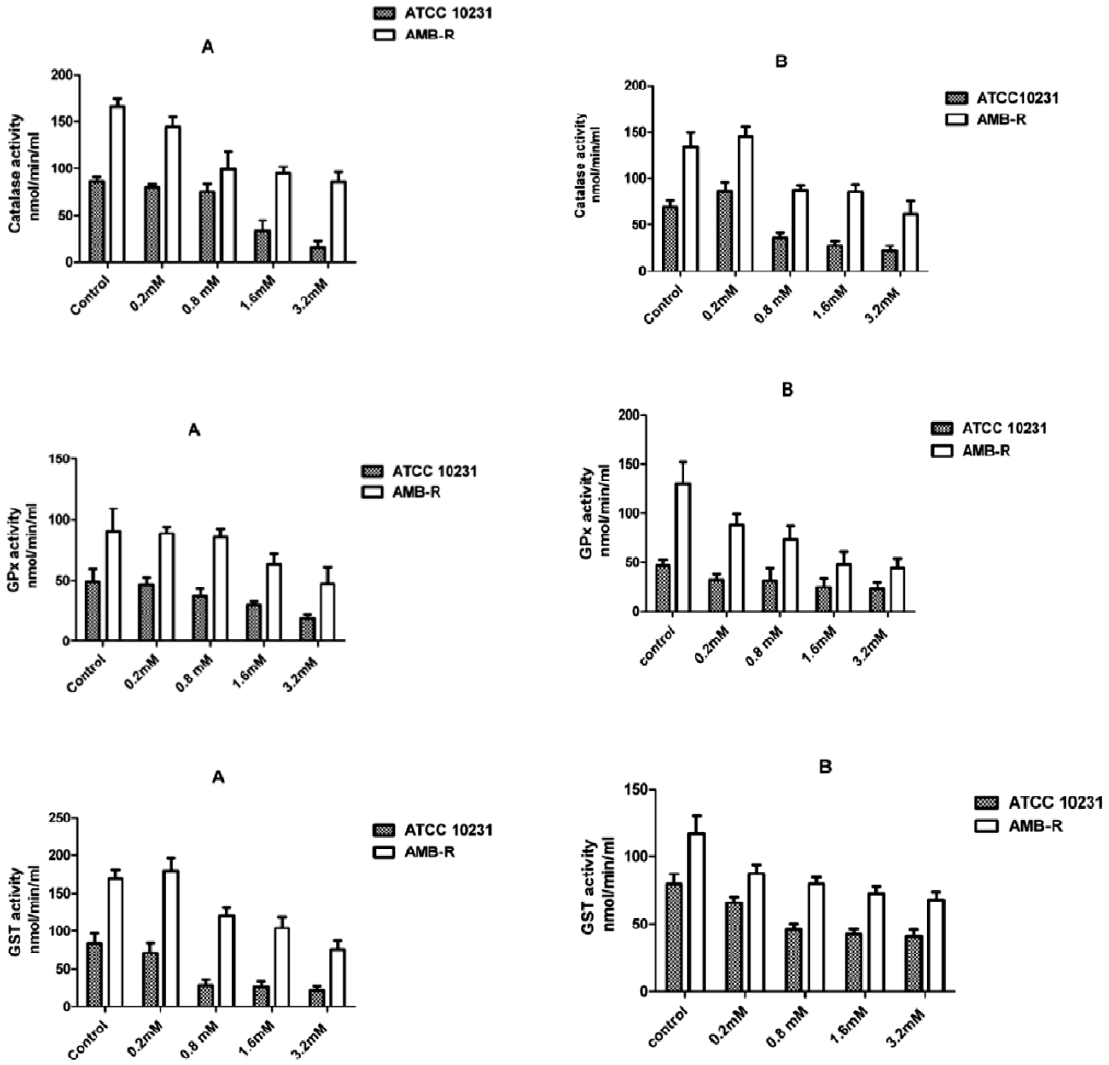


Figure 12. Yeast and hyphal /pseudohyphal form of both parent *C. albicans* and Resistant AMB-R morphological form were challenged to oxidative stress at 0.2, 0.8, 1.6 and 3.2mM menadione. The activities of antioxidative enzymes catalase, glutathione peroxidase and glutathione S transferase were estimated in the cell lysates. The differences between the enzymatic activities are statically significant ($P<0.001$)

4.13. Cell Wall Preparation and Protein Extraction

Cultures of *Candida albicans* were maintained by routine subculture on SDA slants at 28^{o}C. For isolation of cell wall, the yeast cells were mixed with glass beads and vortexed either on a common vortex mixture or in a Beadbeater® for 12-14 cycles 1min each with 2min interval (for cooling). The resultant lysate was observed under phase contrast microscope and no cell was found intact after 10-12 cycles of beating in Beadbeater®. One part of this lysate was also spread on SDA plate where no growth of the fungus was observed after 48h of incubation at 28^{o}C. The cell wall isolated by centrifugation was stored at -80^{o}C.

4.14. Isolation of Covalently Linked Cell Wall Proteins

The cell wall of both yeast and hyphae/pseudohyphae forms of parent *C. albicans* and AMB-R strain was subjected to various chemical/enzymatic treatments. The GPI CWPs were isolated using two methods, chemical, and enzymatic treatment. Hydrogen fluoride (HF)-pyridine was found to be the method of choice for chemical treatment and β-glucanase was used for enzymatic treatment. The cell wall of yeast and hyphal forms of both the strains were run on 12% SDS-PAGE along with marker. Figure 13 represent the different fractions of yeast and hyphal cell wall proteins

4.15. Protein Estimation Using 2D Quant Kit

Protein quantification of all the above samples was done using 2D Quant kit. The principle behind the quantitation is to quantify precipitating proteins while leaving interfering substances in the solution. The assay is based on the specific binding of copper ions to protein. The unbound copper was measured with a colorimetric agent. The quantity of protein in the sample solution was calculated using the standard graph generated. The colour density is inversely proportional to the protein amount in the sample solution. The protein amount estimated in the samples is mentioned later in their respective sections.

4.16. Immunoblotting of GPI Cell Wall Proteins with Pooled Patient Sera and Antiserum Raised in Mice

GPI CWPs were extracted from *C. albicans* cell wall with HF-pyridine and using β glucanase. Various covalently bound cell wall proteins of *C. albicans* are instrumental in fungal adhesion, virulence, and cell wall morphogenesis. After extraction and SDS-PAGE of GPI cell wall proteins with chemical and enzymatic treatment, various protein bands appeared when probed with pooled patient sera and polyclonal sera generated in mice (Figure 14).

4.17. Identification and Characterization of GPI Cell Wall Proteins by MALDI-TOF

Immunogenic protein bands detected on SDS-PAGE single dimension 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 (MASCOT, Aldente. pepMAPPER). A total of seven proteins were identified as GPI cell wall proteins (Figure 15).

The protein sequences were analysed for GPI anchor prediction at C terminal using the software PredGPI. All the proteins were identified as GPI proteins on the basis of software algorithm. PredGPI is a prediction system for GPI proteins which is based on a support vector machine (SVM) for the discrimination of the anchoring signal, and on a Hidden Markov Model (HMM) for the prediction of the most probable omega-site. The omega site has been indicated in bold letter and its position has been given in table 2. The details of GPI prediction analysis has been given in appendix.

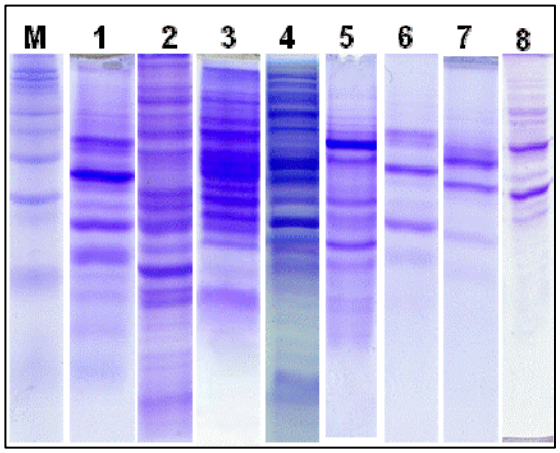


Figure 13. *C. albicans* cell wall profile stained with Coomassie blue of on 12% SDS-PAGE. Strips (left to right): M, standard molecular mass marker; 1, yeast cell wall protein; 2, hyphal cell wall proteins 3, yeast cell wall protein of AMB-R, 4; psuedohyphal cell wall protein of AMB-R, 5; GPI anchored yeast cell wall protein, 6; GPI anchored hyphal cell wall proteis, 7; GPI anchored yeast cell wall protein AMB-R, 8; GPI anchored pseudohyphae cell wall proteins

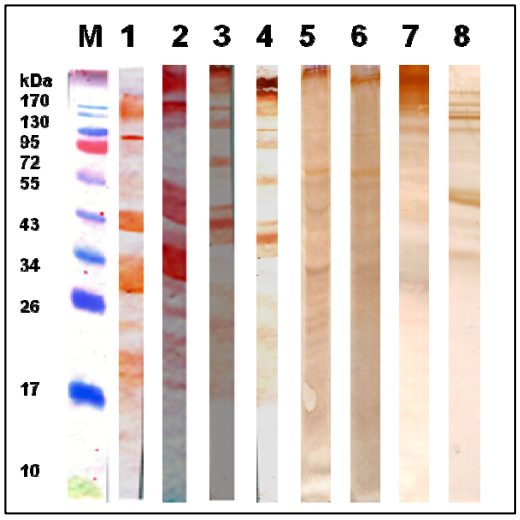
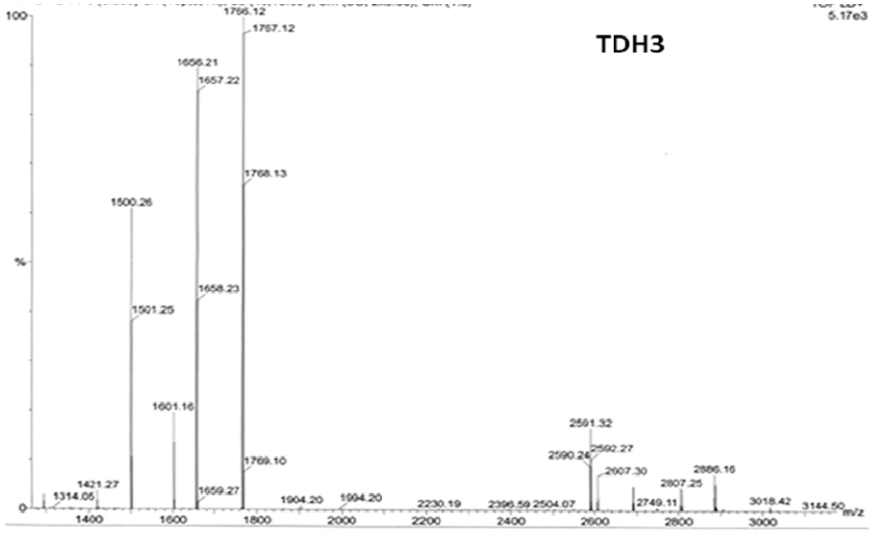


Figure 14. Immunoblotting of GPI anchored cell wall proteins. M; Prestained Marker, Lane 1 and 2; Yeast GPI anchored cell wall protein of *C. albicans* and AMB-R with mice antisera Lane 3 and 4; Hyphal/pseudohyphal GPI anchored cell wall protein *of C. albicans* and AMB-R with mice antisera, 5 and 6; Yeast GPI anchored cell wall protein of *C. albicans* and AMB-R with patient sera, 7and 8; Hyphal/pseudohyphal GPI anchored cell wall protein of *C. albicans* and AMB-R with patient sera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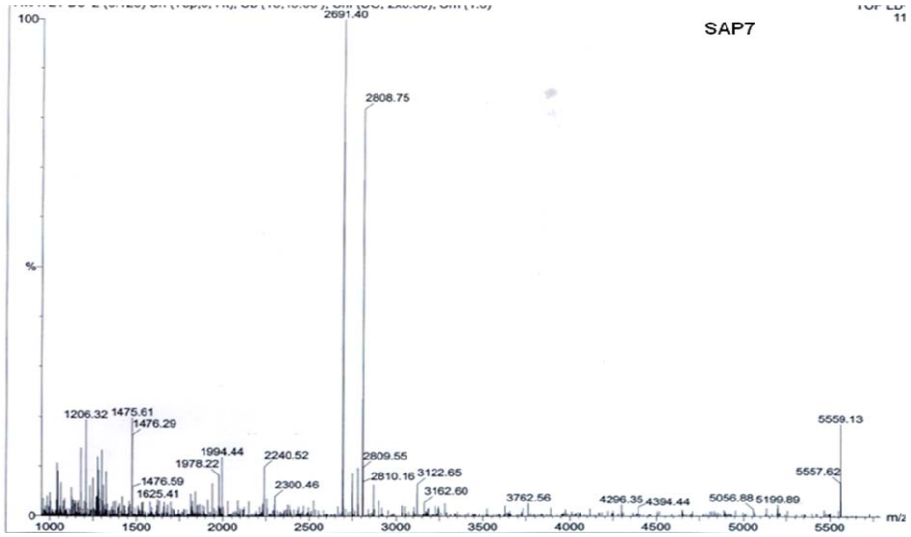
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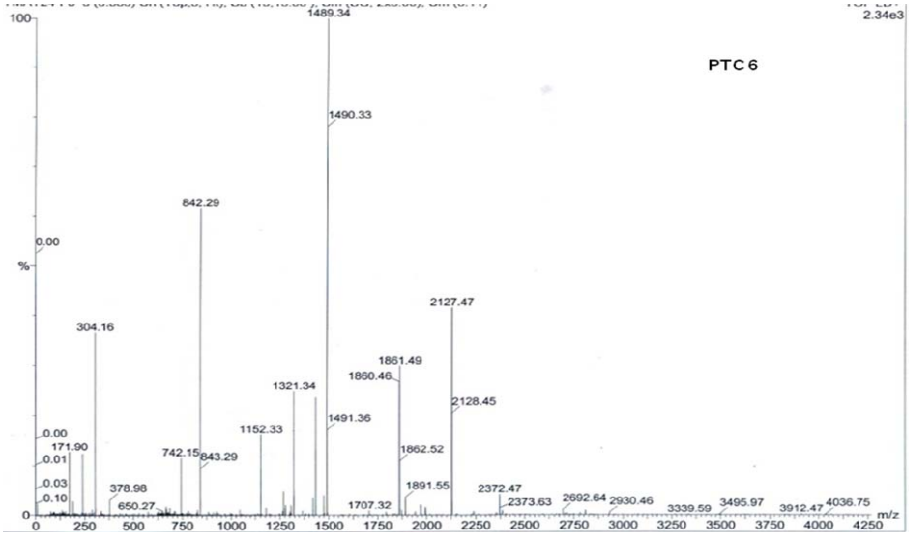
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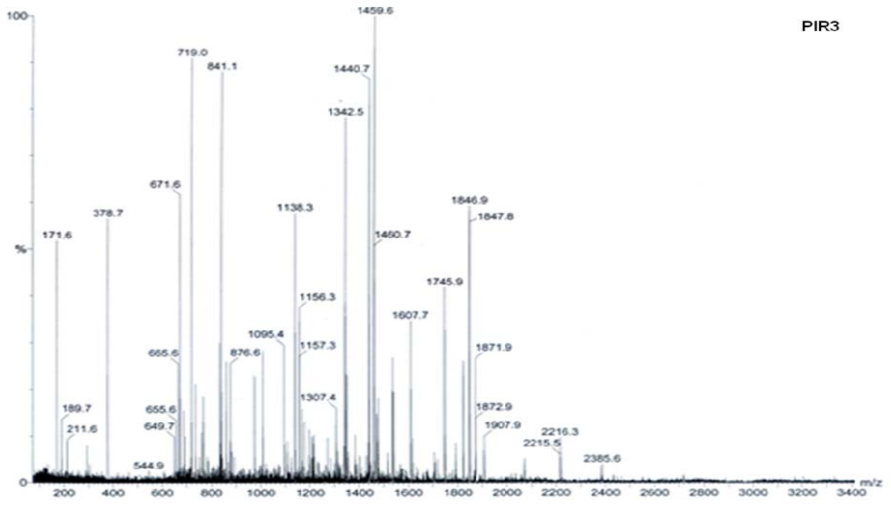
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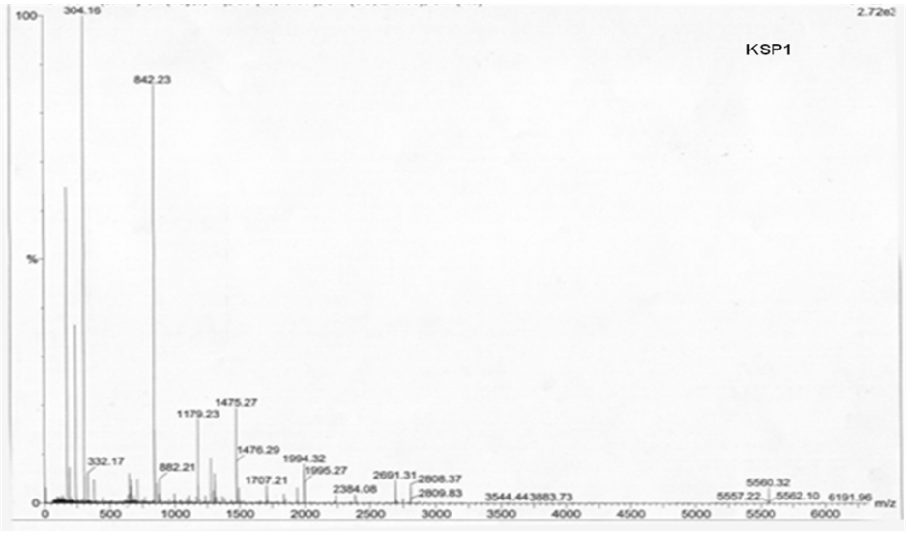
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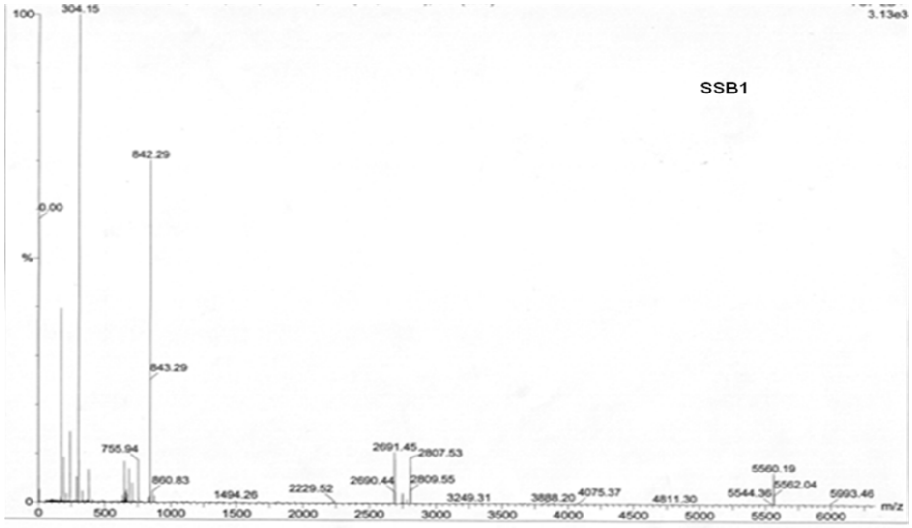
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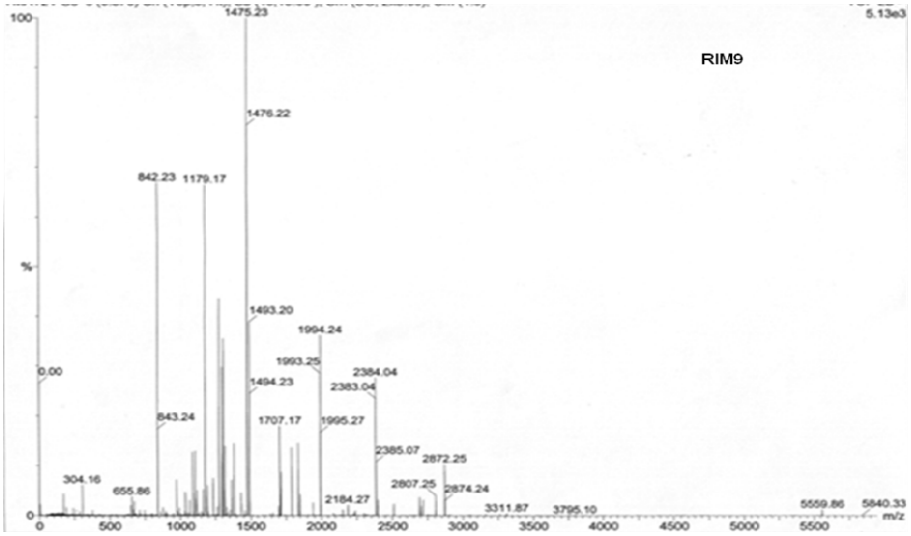

Figure15. MALDI-TOF-MS spectra of *in situ* tryptic digested peptides obtained from GPI anchored cell wall protein SDS PAGE gel, which were immunoreactive with pooled patient sera and polyclonal mice sera, Labelled peptides were used for database searches

Table 2: GPI anchor prediction in identified protein sequences

Protein name	CGD ID	ω-site position	Specificity	GPI anchor sequence
TDH3	**ORF19.6814**	**312**	**64.0%**	L I S **W** Y D N E Y G Y S T R V V D L L E H V A K A S
SAP7	**ORF19.756**	**565**	**84.6%**	A V F **D** L E D H V I S I A Q A A Y N D N H A V V P I E
PTC6	**ORF19.3705**	**407**	**37.3%**	N G W **G** N W P I I D R T G E L R Q A R L D D Y N P R G A R G
PIR32	**ORF19.2783**	**404**	**6.3%**	L Y **D** E P I A F Q C H P V T L D V V E L I E C
KSP1	**ORF19.4432**	**925**	**17.6%**	N **K** S R F N K S I P V G L E L V S S F R K D W C D Y D
SSB1	**ORF19.6367**	**593**	**90.9%**	S A **D** D Y R K A E L A L K R A V T K G M A
RIM9	**ORF19.101**	**326**	**24.4%**	D T **D** D S R R G S S P H E F I E L Q N L R P V

Note: The identified proteins were subjected to BLASTp using The Candida Genome Database (CGD; http://www.candidagenome.org). The CGD ID has been assigned to the best hit protein.

4.18. DNA Fragmentation and Cytokine Assay

Fragmentation of cellular DNA into low molecular weight oligomer is the characteristic of apoptosis. The effect of GPI cell wall protein on J774 macrophage viability was evaluated by agarose gel electrophoresis. Macrophage J774 incubated with three different protein concentrations (20 µg, 40 µg, 60 µg) showed distinct DNA ladder formation with band ranging from 150 to 1000bp while there was no evidence of DNA fragmentation in control cells (Figure 16). The positive control was taken as glucan molecule which also induced DNA fragmentation in macrophages. The GPI cell wall proteins from resistant strain AMB-R induced similar DNA fragmentation in J774 macrophage.

Cytokine level were evaluated using RT-PCR for MIP-2 .IL10, iNOS and IL-12 expression. But there was no difference in expression level of cytokines in presence of parent strain and AMB-R GPI CWPs.

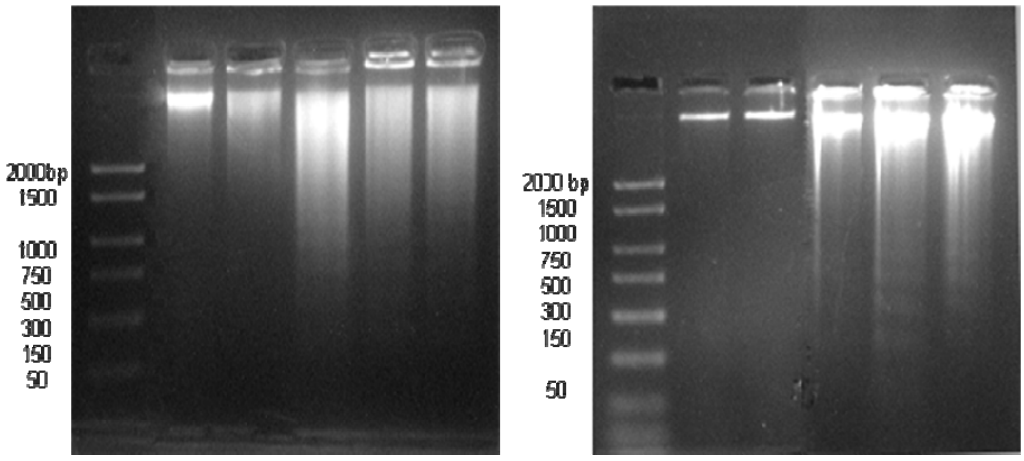

Figure 16. Agarose gel electrophoresis analysis of DNA fragmentations in J774 cells after exposure to GPI CWPs for 16 h. One representative experiment of three is shown. A: ATCC10231 B: AMB-R. lane 1; control J774 DNA,2; J774 DNA in presence of glucan, 3; 20 µg, 4; 40 µg ,5; 60 µg of GPI hyphae/ pseudohyphae cell wall proteins

4.19. Generation and Selection of Monoclonal Antibodies

Mouse myeloma cell line Sp2/O was used for fusion with spleen cells obtained from immunized mice. The fused cells were observed under phase contrast microscope daily to keep track on cell morphology and contaminants. The screening for *Candida*-specific monoclonal antibody producing hybridoma cell lines was started as soon as the hybridoma cells exhibited optimum population of 25-30 cells. During this period, more wells exhibited growth of hybridomas and were included in screening experiment. From this first round of screening, 108 clones showing specificity to the *C. albicans* GPI cell wall proteins of hyphal form were selected. The clones exhibiting high ELISA titer (35 hybridomas) were subjected to single cell cloning. Many clones lost their antibody producing capacity in further sub culturing. After 3-4 rounds of sub-culturing and confirmation by ELISA, ten consistently monoclonal antibody producing clones were identified. These hybridoma clones were propagated in 24 well culture plates and after collection of enough supernatant, the western blot analysis against hyphal GPI cell wall proteins of *C. albicans* exhibited binding of all the clones with different protein bands. Antibodies from one of the hybridoma clones were found to bind with multiple proteins on Western blots (Figure 17). 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 polyclonal sera was also performed in parallel and the clones showing bands common with any of the protein bands identified by patient sera as well as polyclonal sera were selected for further studies. The three such clones were designated as 2A11, 10D2 and 2C1 and selected for further studies.

4.20. Determination of Monoclonal Antibodies Isotype

The isotype of the selected monoclonal antibodies were identified with Isostrip^{TM} mouse monoclonal antibody isotyping kit (Figure 18).

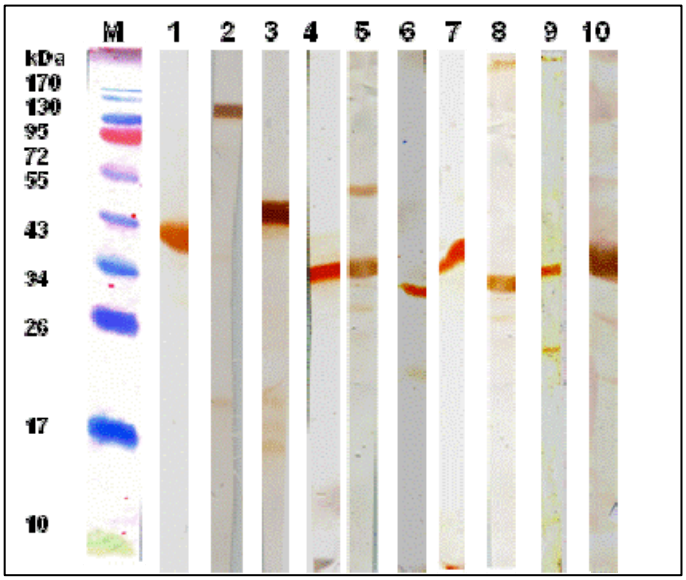

Figure 17. Western blot of positive hybridoma clones.Lane 2 10D2; Lane 4 2C1; Lane 5 2A11

Figure 18. Isotype of monoclonal antibodies 2A11 10D2 and 2C1

4.21. Production of Monoclonal Antibodies Under Serum-Free Conditions

For performing some sensitive experiments where serum proteins interference affects the results, all the three MAbs 2A11, 10D2 and 2C1 were grown under serum free conditions with gradual change of RPMI 1640 medium containing FBS to serum free low protein medium. The monoclonal antibody producing ability of the clone decreased considerably under serum free conditions but was not lost. Approximately 0.5mg/ml MAb was obtained under serum free conditions as estimated after ammonium sulphate precipitation and dialysis.

4.22. Cross Reactivity of MAb 2A11, 10D2 and 2C1 with Other Fungi and Different Strains of *C. albicans*

All the three monoclonal antibodies 2A11, 10D2, and 2C1 were tested for their ability to cross react with cell wall antigens of other *C. albicans* strains, other yeasts, and mycelial fungi. Seven strains of *C. albicans* (4 patient isolates from our lab a 3 typed strains ATCC10231, ATCC14053, MTCC183) and five species of other fungi viz. *Aspergillus fumigatus* (patient isolate), *Trichophyton mentagrophytes, Sporothrix schenkii, Cryptococcus neoformans* (ATCC 66031), and *C. parapsilosis* (ATCC 22019) were tested. MAb 2A11 and 10D2 did not cross react with antigens of fungi other than *C. albicans* (Figure 19) whereas 2C1 showed cross reactivity with yeast like fungi (Figure 20).

4.23. Cross Reactivity of MAbs with Bacteria

To check the cross reactivity of all the three MAbs 2A11, 10D2 and 2C1 against both Gram positive (*Staphylococcus aureus, Bacillus cereus*) and Gram negative (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*) bacteria, total bacterial cell protein was isolated by boiling the cell pellets with SDS PAGE sample loading buffer. Western blot analysis exhibited that all the three MAbs did not cross react with bacterial proteins.

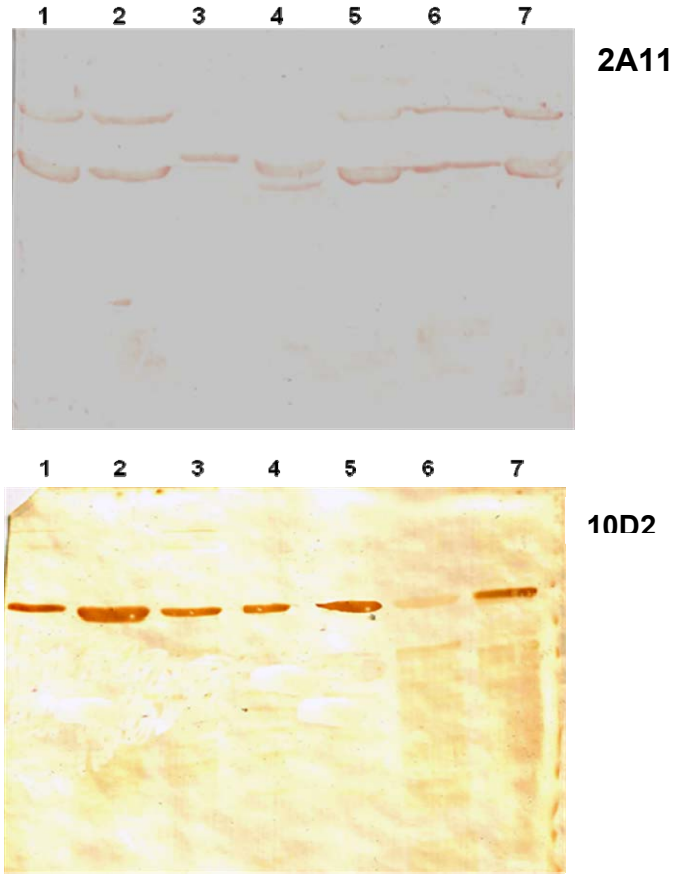

Figure 19. Cross reactivity of MAb 2A11 and 10D2 with different strains of *C. albicans* 1, 2, 3-patient isolates, 4- ATCC14053, 5-ATCC10231, 6-AMBR and 7-MTCC183

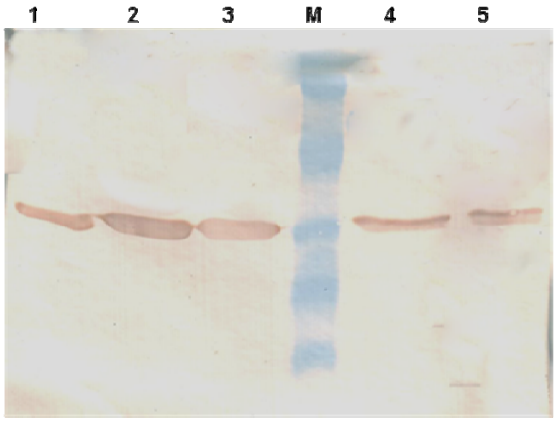

Figure 20. Cross reactivity of MAb 2C1 with yeast form fungi.1; *C. parapsilosis* (ATCC-22019) 2; *C. tropicalis* (ATCC-750) 3; *Cryptococcus neoformans* (ATCC 66031), 4; *C. albicans* (ATCC10231), 5; AMB-R

4.24. Monoclonal Antibody Production by Ascites Growth

Enlarged mouse peritoneal cavity was indication of the formation of ascites in peritoneal cavity. The ascites fluid was collected by tapping after day 10 of inoculation of the hybridoma. An average of about 2-3ml ascites fluid was collected from each mouse in the first tapping followed by few more tapings as soon as the peritoneum cavity got filled with the ascites fluid. Total of 18 ml ascites fluid was harvested from all the mice. The ascites fluid was centrifuged and filtered through 0.22µm syringe filter to remove cells and the cell debris and stored at -20^{o}C until used further.

4.25. Purification of Monoclonal Antibody

Antibody purification from culture supernatant or ascites fluid of MAb 2A11 and 10D2 was carried out using HiTrap Protein G HP column. Ascites fluid or culture supernatant was dialysed overnight in binding buffer to acheive the required pH to increase the binding of the MAb to protein G. The Mabs containing culture supernatant/ascites fluid were passed through the Protein G column and were eluted using glycine-HCl (pH 2.7) and the eluents were collected directly in microfuge tubes containing Tris-HCl (pH 9.0). The purity and the presence of the monoclonal antibody was confirmed by SDS PAGE and the fractions containing pure MAb 2A11 and 10D2 were pooled and stored at -20^{o}C after estimating the protein value. MAb 2A11 and 10 D2 with an average of 1mg/ml (protein value) from culture supernatant and 3.5mg/ml (protein value) from ascites fluid was obtained after purification.

4.26. Assessment of Therapeutic and Diagnostic Potential of Monoclonal Antibodies

4.26.1. Effect of Monoclonal Antibodies on Colony Forming Units (cfu)

MAb 2A11 and 10D2 exhibited potent cidal activity against both *C. albicans* and AMB-R, as significant reductions in the number of cfu compared to controls (without any MAb) as well as with an irrelevant MAb were observed (Figure 21). The MAb 2C1 did not showed good inhibitory activity in both the strains. The optimum dose of MAb 2A11 was found to be 50 µg, where it caused 94.8% reduction in cfu ($P<0.0001$) of *C. albicans* whereas at same

concentration of MAb-10D2 caused 92.5% reduction (P<0.001) and MAb 2C1 exhibited only 39.0% reduction in cfu.

4.26.2. Inhibition of Attachment of *C. albicans* 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. The opsonisation of antibodies reduces the binding capacity of *C. albicans*. An assay was performed to evaluate the capacity of monoclonal antibodies to inhibit the binding of *C. albicans* to plastic culture plates. In the present work MAb 2A11 and MAb 10D2 significantly inhibited the binding capacity of *C. albicans* and AMB-R as compared to MAb 2C1 (Figure 22).

4.26.3. Inhibition of Germination of *Candida* Cells

In order to evaluate inhibition of germination by the monoclonal antibodies, cells of *C. albicans* and AMB-R were incubated with all the three monoclonal antibodies in RPMI 1640 supplemented with 10% FBS at 37^{o}C, 5% CO_2, for 4 h. 2A11 and 10D2 reduced germ tube formation to 45% and 75% as 2C1 reduced 19% germ tube inhibition(Figure 23). As AMB-R has property of reduced germ tube formation under hyphal induction media, the effect of MAbs on AMB-R were less than that of *C. albicans*.

4.26.4. MTT Assay

Fungicidal activity of monoclonal antibodies was determined by MTT reduction assay where a maximum reduction of 91% and 89% in viability was observed for 2A11 and 10D2 when compared with DPBS. 2C1 had 25% reduction of cell viability. A minimal inhibition of 11% and 17% was also observed for the DPBS and irrelevant monoclonal antibody, respectively (Figure 24).

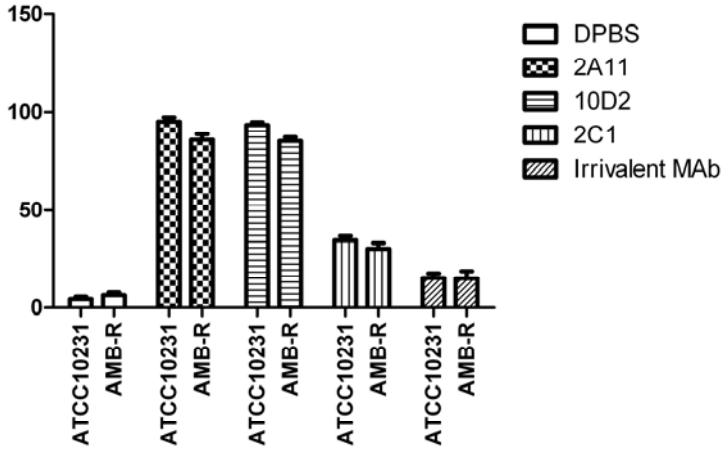

Figure 21. *In vitro* fungicidal activity of monoclonal antibodies against *C. albicans* and AMB-R was measured by the reduction in the number of cfu compared to the control with an irrelevant MAb. Values are means of triplicate determinations ± standard errors of the means. The fungicidal activity of MAb 2A11,10D2 and 2C1 did not exhibited any statistically significant (*P<0.001*) in *C. albicans* and AMB-R. There was significant difference among the MAbs tested.

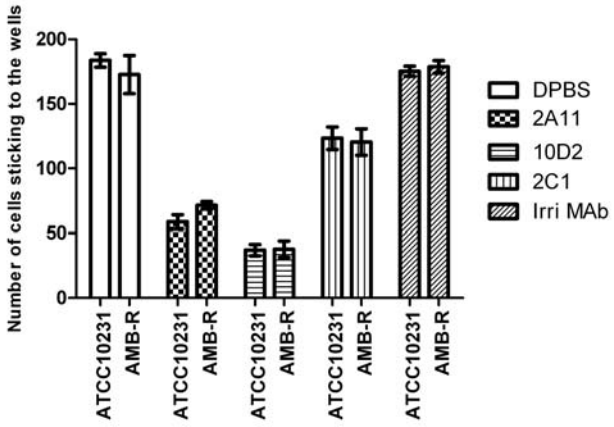

Figure. 22. Exponentially growing cells of *C. albicans* and AMB-R were washed in DPBS and mixed with equal amount of antibody solution (1 mg/ml) such that the final concentration was 2 x 10^3 cells/ml. 100 µl of this was added to ELISA plate (non treated) and kept in incubator for 1h. The wells were gently washed thrice with DPBS and cells sticking to the wells were counted under phase-contrast inverted microscope.

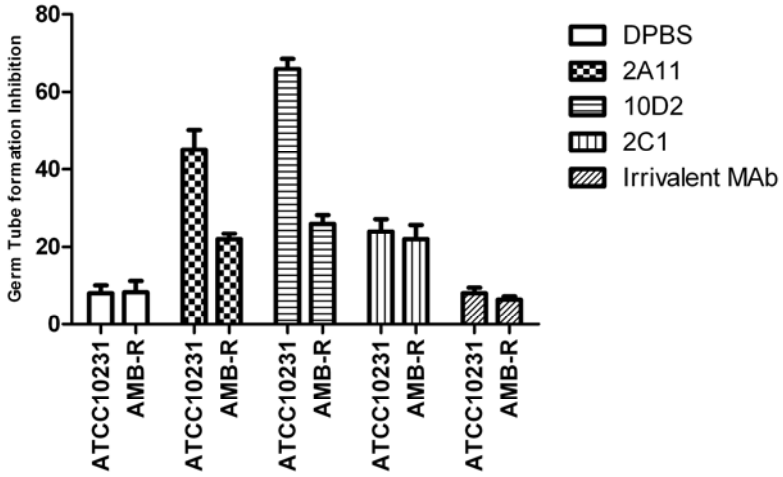

Figure 23. *In vitro* effect of different monoclonal antibodies on germ tube formation capacity of *C.albicans* and AMB-R. Yeast cells were incubated at 37^{o} C with 50 µg of different MAbs in 100 µl RPMI 1640. At selected time intervals, percent germination was calculated. A total of 100 conidia per field were counted at a magnification of X400, and the mean value from three independent experiments was calculated.

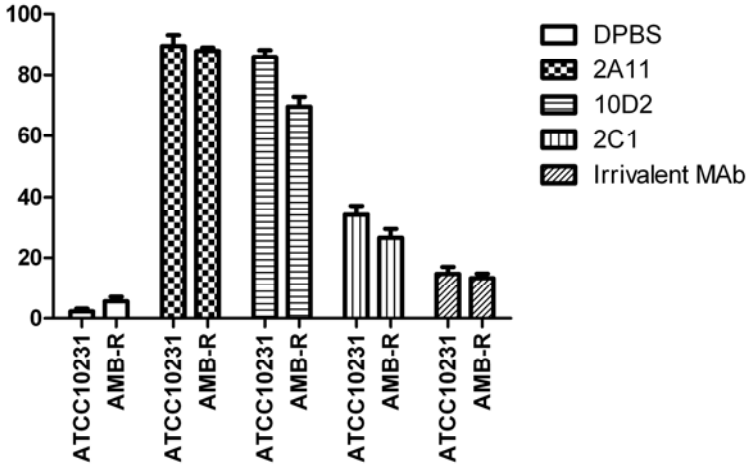

Figure 24. MTT assay for assessment of *in vitro* activity of MAbs. Cells of *C. albicans* were incubated for 16 h, in presence of monoclonal antibodies. After incubation, MTT was added and incubated for another 1h. MTT was converted to an insoluble formazan product that was solublized by addition of DMSO and optical density was read at 550 nm. OD value of DPBS (control treatment) was considered as 100% growth.

4.26.5. Cell-Viability Assay by FACS

In order to confirm the candidacidal activity of monoclonal antibodies, the cells of *C. albicans* were incubated in presence of MAbs for 1h, and then 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 1h, 89% of the cells were found to be dead that were treated with MAb 2A11 where as 81.1% cell death was observed under the influence of MAb 10D2 (Figure 25). There was no significant difference in cell death in AMB-R due to MAb 2A11 and MAb 10D2. Amphotericin B killed 90% of the cells while irrelevant antibody treatment resulted in only 18% dead cells. Growth control (DPBS) had more than 95% cells alive. Data represents the best of the three separate experiments.

4.26.6. Epitope Localization on the Surface of *C. albicans*

Epitope of 2A11 was localized evenly on the surface of both, the yeast and the hyphal forms in both parent *C. albicans* and AMB-R strain (more fluorescence at basal end).The epitope for 10D2 was found to bind evenly with the whole surface of both yeast and hyphae for both *C. albicans* and AMB-R. The cells that burst out exhibited greater fluorescence with 2C1 towards the inner side of the cell indicating that the antigen is present in greater concentrations inside the cell (Figure 26).

4.26.7. Phagocytic Assay

The role of MAbs 2A11, 10D2 and 2C1 in opsonisation and phagocytosis of yeast and germ tubes of *C. albicans* by mouse macrophages was studied using J774 cell line. Yeast cells and germ tubes treated with PBS served as control. Only MAb 2C1 showed significant effect on phagocytosis. After three hours of co-culturing, phagocytosed yeasts and germ tubes were counted under a microscope after fixing and staining the phagocytes. An average of 12-14 yeast cells or germ tubes of parent and AMB-R treated with MAb 2C1 were phagocytosed by the macrophages. Only 4-6 yeast cells per macrophage were seen in case of PBS treatment whereas 10-12 germ tubes per macrophage were seen in control. Percent phagocytosis was calculated

by counting the number of cells present within the phagocytes per 100 macrophages (Figure 27). About 71% phagocytosis was observed in case of yeast cells of both parent and AMB-R strain treated with MAb 2C1 as compared to control where only 21% phagocytosis was observed in case of parent *C. albicans* and 13% phagocytosis was observed in AMB-R. In case of germ tubes treated with MAb 2C1, 84% phagocytosis was recorded as compared to those treated with PBS (24%).

4.26.8. Protection Against Experimental Murine Candidiasis by MAb 2A11 and 10D2

As MAb 2A11 and 10D2 showed its potential as therapeutic antibody in biological activities like germination assay, phagocytic assay, *in vitro* inhibitory activity, MTT assay and FACS analysis therefore these two MAbs were used for *in vivo* evaluation for determining therapeutic efficacy. *In vivo*, 2A11 and 10D2 exhibited good antifungal activity in a mouse model of candidiasis. A reduction in cfu of more than 4 $\log_{10}$ units was observed in kidney tissue of experimental mice compared to control in case of MAb 2A11 whereas reduction in cfu of more than 2 $\log_{10}$ units was calculated in case of 10D2 (Figure 28).

4.27. Assessment of *In Vivo* Diagnostic Potential of Monoclonal antibody

In vivo diagnostic potential of the monoclonal antibody 2C1 was assessed in an animal model of *C. albicans* using BALB/c mice. The mice were injected with a sub lethal dose of *C. albicans* yeast cells and mice without any infection served as control. After the establishment of infection, serum from each mouse was collected at an interval of three days and presence of the fungal antigens was assessed by using MAb 2C1. Since the model is a mouse model and the hybridoma was developed by the fusion of splenocytes from the same species, the secondary antibody used against MAb 2C1 may also bind with antibodies present in the mice serum thus giving false positive results. This problem was overcome by coupling biotin to MAb 2C1 and detection was done using HRP conjugated to streptavidin. Streptavidin-biotin interactions are one of the strongest and stable interactions thus increasing the reliability of the

test. Using ELISA, the *C. albicans* antigens could be detected significantly in four of the six mice. No antigen could be detected in control mice. Thus the detection efficiency of the MAb 2C1 was found to be 51% in the present study.

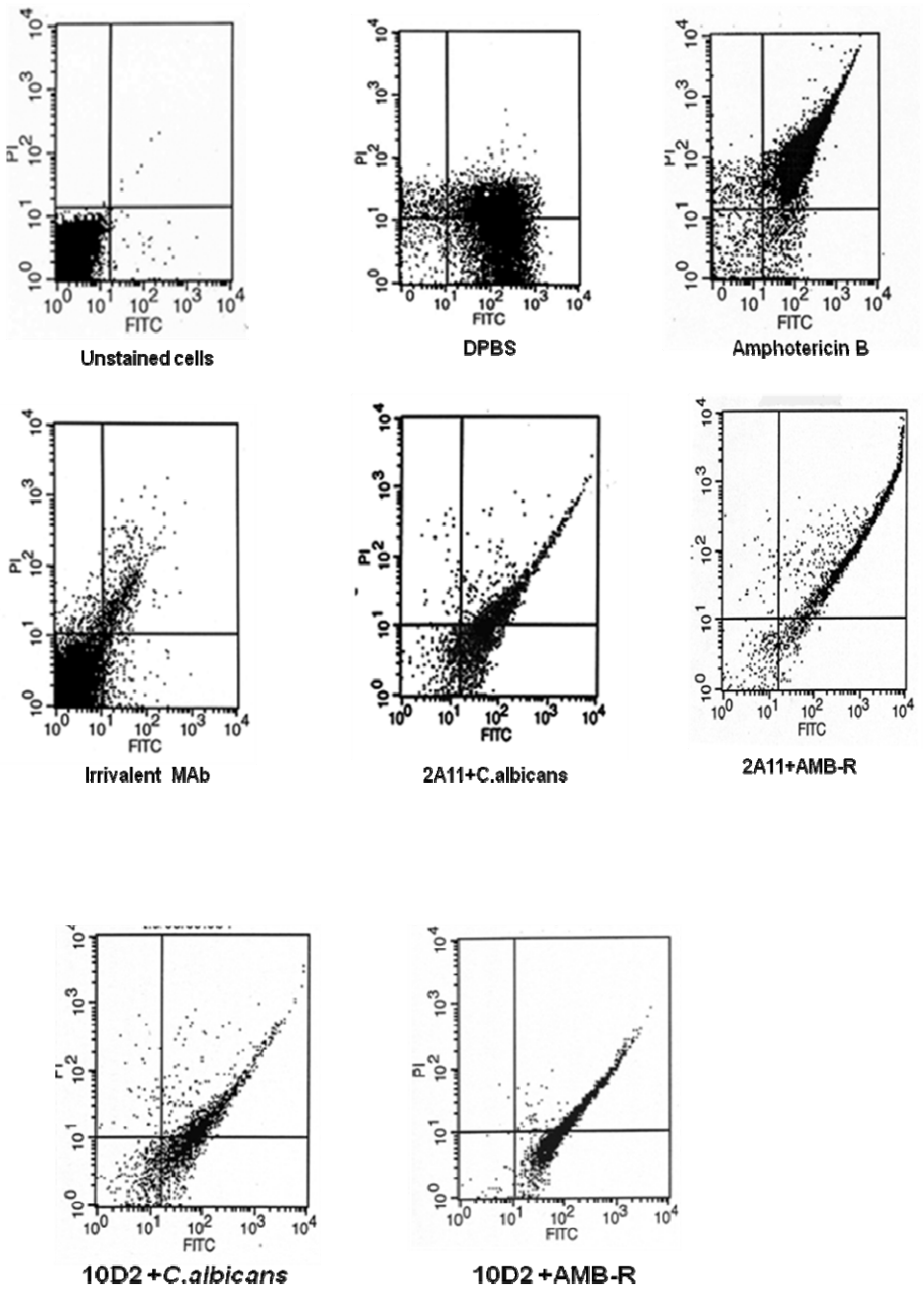

Figure 25. Exponentially growing *C. albicans* cells were exposed to all MAbs at concentration of 100 µg (2A11, 10D2 and 2C1, irrelevant MAb), with DPBS only as a negative control and with AmB as a positive control for duration of 18 h at 37^{o}C, differentially stained for live and dead cells and analyzed on flow cytometer.

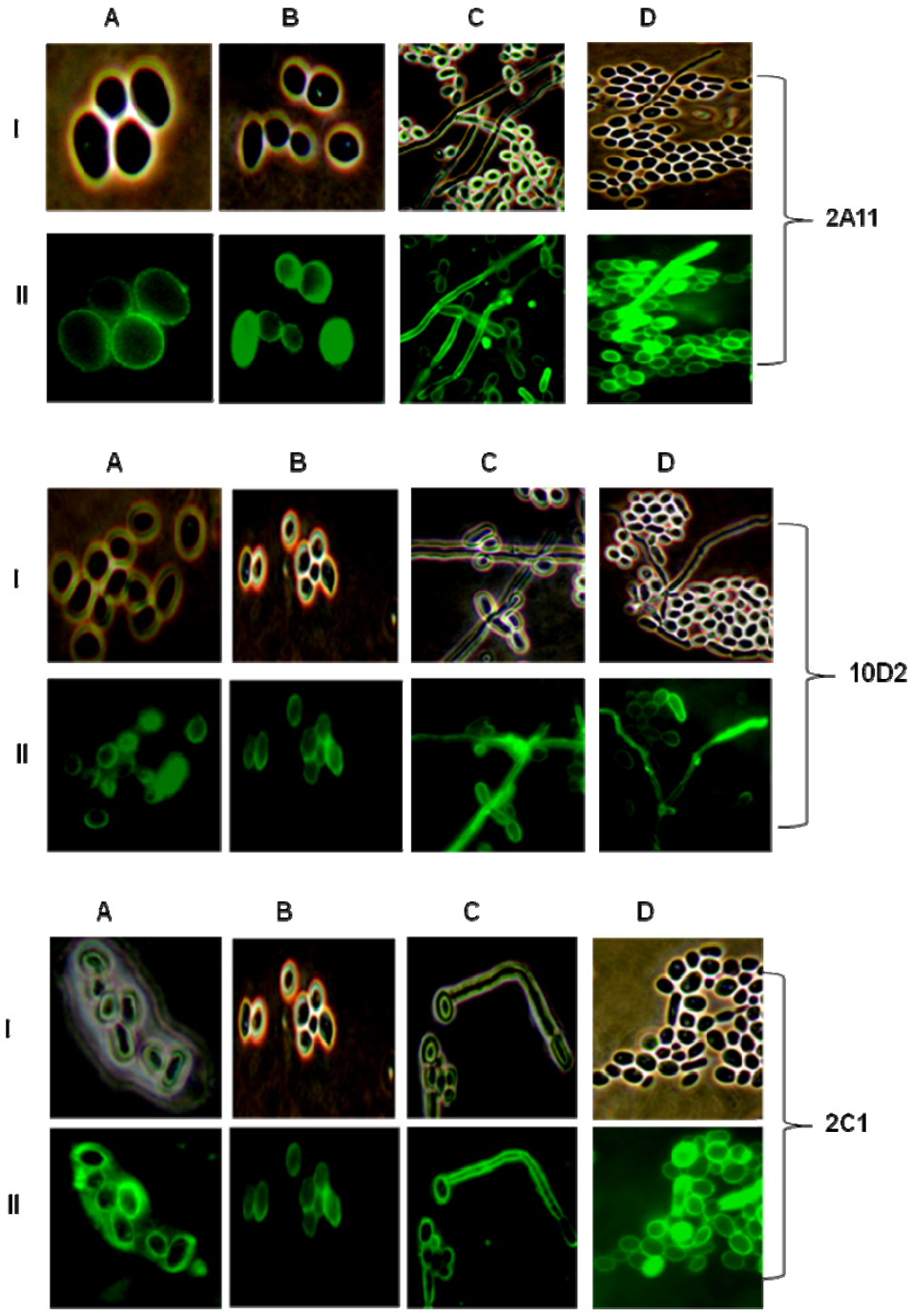

Figure 26. Epitope localization of the antigens recognized by the three MAbs 2A11, 10D2 and 2C1 using indirect immuno-fluorescence microscopy I. DIC of cells II. immuno-fluorescence in cells. Control antibodies used in these experiments did not show any fluorescence. No irrelevant binding of secondary antibodies was observed.

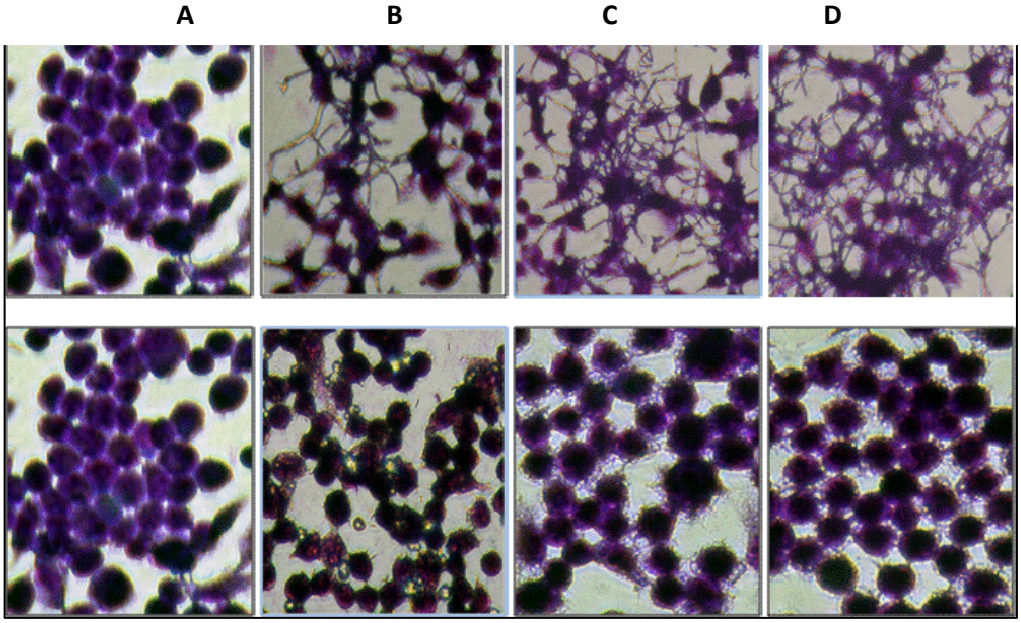

Figure 27. Phagocytosis of *C. albicans* yeast cells and germ tubes by mouse macrophage cells J774.A, resting macrophage B. untreated yeast and germ tube of *C. albicans* C. MAb treated *C. albicans* D. MAb Treated AMB-R.

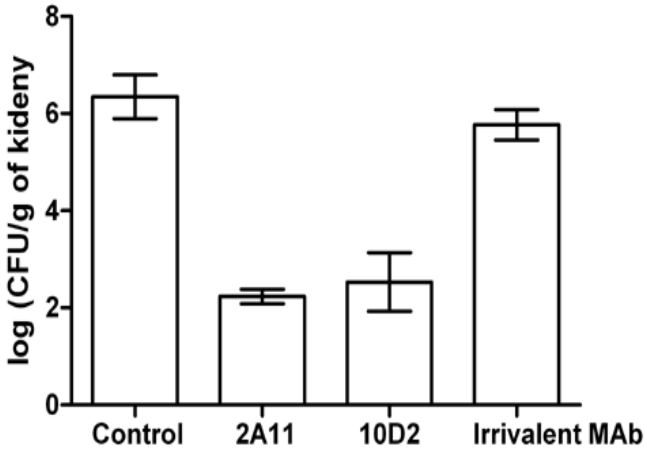

Figure 28. Effect of MAb 2A11 and MAb 10D2 on the reduction in cfu from kidney tissue of experimental mice. A reduction in cfu of >4 $\log_{10}$ units and >2 $\log_{10\ units}$ were observed in kidney tissue of BALB/c mice challenged with *C. albicans* (2.0 x 10^5 cells per mouse). MAb 2A11 and MAb 10D2 were administered intraperitoneally at 200 µg of test antibody (1 mg/ml) intraperitoneally 4h before challenge (with *C. albicans*) and six successive 100 µg doses at 1,2,3,4,6 and 9 days post challenge. The reduction in cfu was significant ($P<0.001$).

4.28. Detection of Antigens Recognized by MAb 2C1 in *Candida* Positive Patient Sera Using ELISA

The ultimate goal of *C. albicans* antigen detection by MAb 2C1 was to check its diagnostic ability during the course of infection in patients suffering with candidiasis. For this purpose fifteen patient (*Candida* positive) sera and nine sera from healthy donors were screened for the presence of *C. albicans* antigens. Here TMB was used in place of OPD due to its higher sensitivity in ELISA. ELISA titre in serum from healthy donors was considered as control to fix the base line for OD values. In eleven out of eighteen patient sera samples, antigen could be detected in the present study (56.8%).

4.29. Peptide Synthesis and Its Evaluation *In Vitro*

Eight peptides was synthesized and checked for their purity and mass spectrum using HPLC and Mass Spectroscopy (Figure 29).

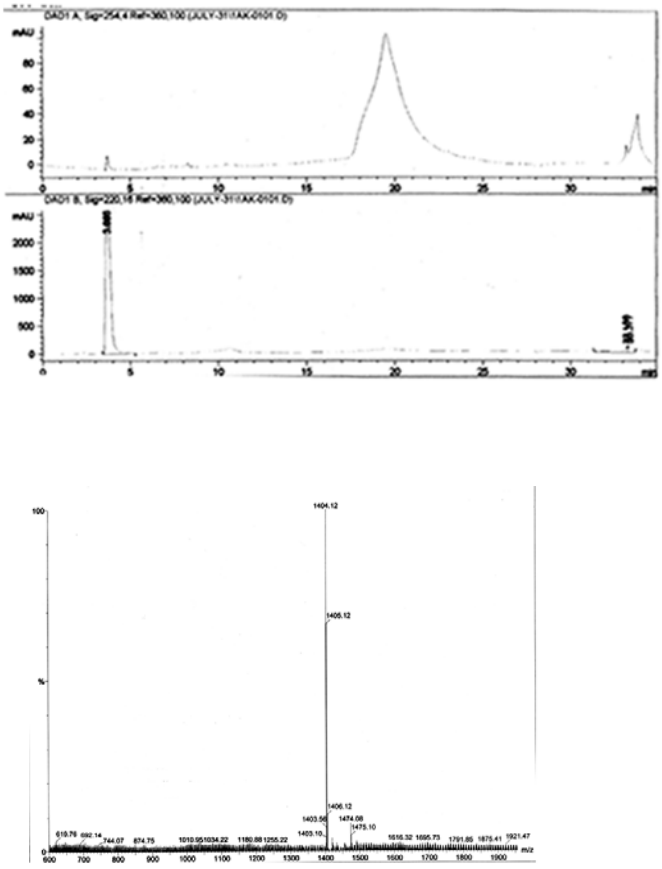

Figure 29. Representative profile (HPLC and MALDI MS) of a synthesized peptide.

Table 3. MIC and IC_{50} values of the eight paratope derived peptides.

	MIC(µg/ml)		IC50(µg/ml)	
Peptide (Sequences)	*C. albicans* ATCC10231	AMB-R	*C. albicans* ATCC10231	AMB-R
1. QIVLTQSPAIMS	>200	>200	>200	134.56
2. GSSPRLLIYDTS	>200	>200	>200	120.54
3. TSYSLTISRVEA	>200	>200	>200	112
4. IFSFLLISASVI	>200	>200	179.44	187.8
5. SPRFLIYDTSNL	>200	**118.92**	>200	94.50
6. AAPTVSIFPPSS	**73.96**	180	30.84	116
7. GGASVVCFLNNF	>200	>200	134	102
8. NSYTCEATHKTS	>200	>200	>200	>200

The synthesize peptides were screened for their *in vitro* activity in *C.albicans* and AMB-R. The MIC and IC_{50} was calculated and shown in Table 3. Peptide 6 was more active in *C. albicans* as compare to AMB-R Whereas Peptide 5 showed more activity in AMB-R than its parent *C. albicans*. The other peptides could not show any effective in vitro activity in both the strains tested.

Discussion

Opportunistic fungal infections represent today a serious and not-yet solved health problem in both developed and developing countries. Several factors have contributed to this increase, such as abuse of antibiotics which has favored the emergence of fungal resistances, immunosuppresion originated by cancer therapy and transplantations, and the progression of HIV infection to AIDS. The use of catheters and protheses, together with surgical techniques and the augmentation of Intensive-care units, contribute also to the expansion of these infections (258,259). Among the opportunistic pathogens, *Candida albicans* is the single most common fungal species causing nosocomial infections worldwide causing considerable morbidity and mortality. Basically a commensal, *C. albicans* can cause a variety of infections ranging from superficial muco-cutaneous infections to life threatening systemic infections (1). The therapy of *C. albicans* infections is facing challenges like relatively lesser number of antifungal agents, increasing incidences of antifungal resistance among pathogenic fungi and also due to lack of reliable, specific and rapid diagnostic methods against this pathogen. Fungi being eukaryotic organisms share many of their biological processes with humans therefore the existing antifungal drugs can cause toxicity when used for treatment (15). The most important and significant area of research would be the development of new antifungal agents with wide spectrum cidal activity and minimum toxicity. One of them seems to be through the development of monoclonal antibodies of therapeutic/diagnostic value, their paratope derived peptides and identification of new targets to overcome the problem of antifungal resistance. Fungal cell wall provides an opportunity for researchers to explore new drug targets due to its unique and complex nature and its importance in pathogenesis and drug resistance in fungi. Hence targeting the molecules responsible for the cell wall complexity may open new leads in antifungal therapy.

5.1. Generation and Characterization of Amphotericin B Resistant Strain

Amphotericin B, a polyene antifungal, once considered the gold standard for the management of a number of fungal infections has its limitations in the form of severe side effects such as bone marrow depression, nephrotoxicity. Further, after decades of continued use, *Candida* species have started showing resistance to amphotericin B. There is lack of understanding associated characterization and

relevance of amphotericin B resistance till now. In case of amphotericin B therapy, the continuous use of the drug exerts selective pressure which may result in a shift towards isolation of more inherently amphotericin B-resistant species. Probably this is the right time to understand the resistance mechanism of amphotericin B in order to differentiate between drug-susceptible and resistant isolates for more safe and effective antifungal therapy. Acquired resistance to amphotericin B in *C. albicans* has been reported in literature along with azole resistance. *Candida* species like *C. lusitaniae* and *C. guilliermondii* frequently develop resistance to amphotericin B and there are few reports on amphotericin B resistance in *C albicans* as well, which is alarming (261,262). Microarray reports on gene expression in AMB resistant strain highlighted genes associated with resistance (263). Natural and acquired drug resistance is an evolutionary process that enables a pathogen to survive and reproduce in the presence of drug.

In this study, serial passaging of *C. albicans* in amphotericin B containing media might have induced strong selective pressure to acquire irreversible and stable resistance that was indicated by enhanced MIC which has been considered as an indicator of acquired resistance. The acquired resistance in pathogens has often been associated with transient resistance phenomenon where the organism reverts to it susceptible form in the absence of drug pressure (264). To determine the stability of amphotericin B resistance, MIC was determined at regular intervals up to 60 days in the absence of amphotericin B. There was no change in MIC which confirmed the stability of the acquired resistance in *C. albicans* indicating the development of amphotericin B resistant *C. albicans* strain which was designated as AMB-R. The resistance in this newly developed strain of *C. albicans* AMB-R was further confirmed in a mouse model of systemic candidiasis where amphotericin B treatment did not reduce the load of pathogen significantly in kidney tissue of mice infected with the newly developed AMB-R compared to mice infected with the parent strain of *C. albicans* where it was significant (100%) (Table 1) thereby indicating an acquired, enhanced and sustainable resistance in the newly developed AMB-R strain.

Time kill assay is a method of choice to study the effect of an antifungal agent where the rate and extent of fungal killing is determined. Time kill studies include

the dose response curves and time course of activity and explain the nature and extent of killing of fungi over a time period by calculating the cfu reduction at different time intervals (265). This can be utilized as important tool to study the extent and nature of resistance developed in fungi against antifungal agents. Both parent *C. albicans* and its derivative AMB-R were incubated for 48h at different MICs and the cfu were compared at different time intervals (Figure1). Due to the cidal nature of amphotericin B, there was gradual reduction in cfu of the parent *C. albicans* whereas in AMB-R strain, no significant reduction in cfu was observed even at 2xMIC which indicated that the resistance level of AMB-R can increase further if continued under drug pressure for longer period. There was no significant reduction in cfu at different time point in AMB-R which indicated that the nature of resistance in AMB-R is inherited and not due to transient resistance/ enhanced tolerance capacity against drug.

During the development of amphotericin B resistance, *C. albicans* was under continuous exposure of drug which might have induced changes at morphological, biochemical as well as genetic level in order to counter attack the drug pressure. In present study, there was a distinct morphological change in the resulting AMB-R strain when compared with its parent *C. albicans*. In the presence of hyphal inducing medium and other favorable conditions like pH and temperature, *C. albicans* converted itself to hyphae while its derivative AMB-R to pseudohyphae. Both pseudohyphae and hyphae are morphologically distinct. In pseudohyphae, each cell to cell junction is constricted and the diameter between cell wall is wider in the middle than the ends. In contrast, hyphal cell walls are parallel without any septal junction constrictions. The changes in morphology of *C. albicans* were observed by fluorescence microscopy using Calcofluor white (fluorescent dye which binds to chitin of fungal cell wall). The morphological changes in AMB-R may provide reasons/consequences of amphotericin B resistance on *C. albicans*.

The conversion of yeast to hyphae in *C. albicans* starts with initiation of germ tube formation. Effect of amphotericin B on germ tube formation in *C. albicans* is well known (183). Germ tube formation assay is a quantitation assay to measure the yeast-to-hyphal transition on the basis of glucan synthase inhibition and resistance to amphotericin B leads towards differential regulation of β-1,6 glucan pathway

genes (1). In the present study, the germ tube formation capacity of both parent and AMB-R strains of *C. albicans* was compared at different concentrations of AMB. As the activation of glucan synthase is associated with the extensive synthesis of cell wall during the formation of hyphae, the inhibition of germ tube formation may be due to inhibition in glucan synthesis. The resistant strain AMB-R was compared to its parent strain for the glucan synthase activity. The 1,6-β glucan signals consistently increased over time (up to 4h) in both the strains but it was greater in the parent strain as compared to AMB-R. Thus the reduction in germ tube formation capacity of AMB-R may be correlated with the reduced glucan synthase activity in AMB-R. The cell wall is a dynamic structure constantly changing in response to environmental signals. Cell wall component, such as glucan, may physically interact with the antifungal and inhibit penetration to the site of action and may lead to reduced susceptibility. So it may be possible that during the induction of AMB resistance, the drug may alter glucan synthesis leading to its reduced capacity of germ tube formation.

Ergosterol is a major component of the fungal cell membrane which helps in maintaining structure and regulates the efflux and influx of the intra/extracellular components. Amphotericin B acts on cells by binding to ergosterol leading to creation of pores through which intracellular constituents leaks and protons enter the cell. The combined effect of these two processes is the acidification of the cytoplasm and death of the cell. Among several clinical isolates of *Candida,* amphotericin B resistance has been correlated with decreased level of ergosterol (266). Mutations that lead to the production of unusual sterol types alter the stereochemistry of membrane ergosterol, and/or decrease the overall quantity of ergosterol in the cell membrane may result in diminished amphotericin B binding capacity and thus lead to decreased efficacy (267). Reduction in sterol level for increased growth in the presence of amphotericin B has been reported (268, 268, 269). It is also reported that azole mediated amphotericin B resistance might be due to changes in membrane composition causing cell wall alteration that affect accessibility of amphotericin B and change cell morphology (270). In the present study, the ergosterol content was estimated in both parent *C. albicans* and AMB-R

strain. The present results also suggest that primary or acquired resistance to amphotericin B may be explained by reduced ergosterol content.

Alteration in the ergosterol biosynthetic pathway is resultant of modification in genes involved in ergosterol biosynthetic pathways. One of the ergosterol biosynthesis pathway genes *ERG11* has been studied for mutations at genetic level which results in polyene resistance among *C. albicans* (271). In the presence of amphotericin B *in vitro*, Sanglard *et.al* reported the development of a mutant with defects in the *ERG11* gene (272). The results indicated changes at nucleotide level which is in similarity with azole resistance where the mutation in ergosterol synthesis pathway genes including *ERG11* modifies the target sites for the azole activities. Amphotericin B resistant strain exhibited over expression of the ergosterol biosynthesis genes *ERG5*, *ERG6* and *ERG25* which encode enzymes that represent critical steps in ergosterol biosynthesis pathway and results in alternate sterol production (271). In another report, an *in vitro* exposure of *C. albicans* leading to resistance to fluconazole was found to be associated mainly with up-regulation of *ERG11* gene (272). Likewise an over expression of *ERG11* gene, in case of AMB-R in the present study, can be correlated with depletion of ergosterol content which may be one adaptive mechanism during the development of resistance. In the present study, *ERG11* and *CSP37* genes were selected to identify mutations if any at nucleotide level (Figure 10). The cell surface protein *CSP37* has been involved process of adherence and attachment to host tissue. The presence of *CSP37* gene on cell surface comes directly in contact with external environment and can modify the behavior of *C. albicans* accordingly. In AMB-R strain, mutations were observed in *CSP37* also. The observed mutations may provide important leads in order to understand the resistance mechanism and consequently provide new drug targets in drug resistant isolates.

Being important as virulence factors secreted aspartyl proteinases (Sap) can be important parameter to understand development of drug resistance and virulence as some antifungal exerts their influence the activities of secreted aspartyl proteinase. Wu *et al* (273) examined the production of Sap after developing *in vitro* resistance against fluconazole and found that exposure to sub inhibitory concentration of fluconazole resulted in enhanced extra cellular production of Sap.

Earlier reports have shown enhanced production of Sap in azole resistant *C. albicans* isolates from a patient infected with HIV after growth under the influence of sub inhibitory concentration of fluconazole (264, 265). Lenkey *et al* (275) also reported that a fluconazole resistant *C. albicans* strain which was developed from a clinical isolate had also enhanced Sap activity than its parent. The Sap activity in the presence of fluconazole and voriconazole has been increased in proportion to the drug concentration. The high concentrations of drugs caused an increased Sap activity in resistant isolates (274).In the present study, secreted aspartyl proteinase in resistant strain AMB-R was compared with its parent strain *C. albicans*. The results indicated significant increases in Sap activity in the resistant strain AMB-R as compared to its parent *C. albicans* strain. There are possibilities of an efflux mechanism in case of amphotericin B (like fluconazole) that regulates the transport of Sap into membrane-bound vesicles, and up-regulation of such transport explains the increased Sap activity in resistant isolates which need to be investigated further.

Extracellular phospholipases are considered to play a significant role in the pathogenesis. It is also known that clinical isolates of *C. albicans,* higher level of extra cellular phospholipase activity correlate well with a number of other pathogenic attributes such as adhesion, invasion of host tissue and increased germ tube formation. Mutant deficient in producing extracellular phospholipase has been more susceptible to amphotericin B deoxycholate (276). There are not many reports stating the effect of AMB resistance on extracellular phospholipase activity. Both parent and AMB-R strains were screened for extracellular phospholipase activity. The phospholipase activity of the isolate was interpreted positive when a precipitation zone was visible around the growth. Phospholipase activity was measured by dividing colony diameter by the diameter of the precipitation zone (Pz) around the colony formed on the plate. The enhanced activity of extracellular phospholipase can be an indication of increased resistant of *C. albicans* towards AMB.

Evidences from several studies have shown that killing of fungi can result from oxidative damage by amphotericin B (277). Amphotericin B induced oxidative stress on fungal cells is an important factor for the cidal activity of the drug and

channel (pore) formation in cell membrane that subsequently causes cell disruption. Cell membrane damage is due to the formation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals that results in membrane disruption and cell death through membrane lipid peroxidation (278). Oxidative changes within the fungal cell may also play a role in polyene resistance among *Candida* species. As the ergosterol synthesis in the cell membranes is oxygen-dependant, alterations in cellular oxidative processes may lead to decreased ergosterol production and subsequent resistance to amphotericin B. There are earlier reports indicating that resistance of fungal cells to oxidative damage could contribute to their decreased susceptibility to amphotericin B (277). In the present study, a decrease in ergosterol level in AMB-R was observed, therefore its correlation with the oxidative tolerance capacity in AMB-R and its parent *C. albicans* was examined on the basis of antioxidative enzymes activities. As the cellular oxidative processes inside the fungal cell may affect the efficacy of amphotericin B, antioxidant enzymatic defence mechanism was studied in AMB-R as compared to its parent *C. albicans* in order to explore the behaviour of AMB-R in the presence of oxidative agents like H_2O_2 and menadione. Menadione is a cytotoxic quinone that generates superoxide anion which is a member of reactive oxygen species (ROS) group. ROS attacks all the essential cell components like DNA, proteins and lipids (278). To mitigate and repair the damage caused by ROS, cells have developed complex antioxidant responses. These antioxidant responses include small molecules like glutathione and some antioxidative enzymes like catalase, glutathione peroxidase, glutathione S transferase among others (279). In the present study, both the morphological forms of parent *C. albicans* and AMB-R were compared for anti oxidative enzymes activities (Figure 11, 12). Catalases protect the cells from oxidative damage by converting H_2O_2 into $2H_2O$ and O_2. An increased catalase activity in *Candida* sp. has been suggested as a mechanism that reduces amphotericin B activity. The present study also supports the earlier reports as there was an increase in catalase activity in AMB-R strain as compared to parent *C. albicans*. Further, the role of intracellular glutathione in oxidative stress was also studied to compare the intracellular glutathione enzymes levels in AMB-R and parent *C. albicans*. Glutathione peroxidase (GPx) plays an important role in

scavenging H_2O_2 generated through respiration in mitochondria. Depletion of glutathione leads to mitochondrial damage. Glutathione S-transferses are detoxifying enzymes that bind covalently/non-covalently to drugs to remove them. The level of both the enzymes was found to be increased in the resistant strain AMB-R as compared to the parent strain. Increased level of all the three antioxidative enzymes in both yeast and pseudohyphal of AMB-R indicated their role in the adaptation of the organism to the continuous exposure of amphotericin B. Kimura (1995) showed that intracellular glutathione plays an important role in the adaptive responses in *S. cerevisiae* to oxidative damage (281). As shown in various research works, exposure of organisms at lower concentration of oxidizing agents offers better adaptation at higher concentration by inducing antioxidative enzymes (282). Cross protection to oxidative agents can also been observed where pre incubation of organism to one oxidative agent provides protection in oxidative damage from other oxidising agents (279). In the present study, it seems that the resistance to amphotericin B provides protection to AMB-R from H_2O_2 and menadione (various concentrations) by increase in level of antioxidative enzymes. The oxidative stress responses in resistant strain AMB-R may be of clinical interest for proper understanding of the invasion and colonization of host tissues by pathogens as well as during the respiratory defensive mechanisms triggered by phagocytes.

5.2. Identification and Characterization of Covalently Linked Cell Wall Proteins

The cell wall is the outer most structure of all the fungi that provides a specific shape and strength and protection to the organism/pathogens which in turn enables them from their existing adverse environment. Further the cell wall comes in the first contact between pathogenic (opportunistic/true) fungus and human/host tissue which is attributed to fungal cell wall components. The major part of the cell wall component is made up of covalently linked proteins and GPI cell wall proteins form the major part of the latter. Therefore the present studies were focused on the GPI CWPs of *C. albicans* which is an endogenous and opportunistic pathogen of human beings. *C. albicans*, being polymorphic fungi, present in both yeast form or as hyphae/pseudohyphae. Both types of morphology may be present in infected host

tissue and it is possible that both may play important roles in the pathogenesis of *C. albicans* (283). Hyphal growth may be more important for the pathogenesis of the fungi as hyphae adhere more strongly to mammalian cells, promote tissue penetration and provide a mechanism to escape the attack by macrophages. This yeast to hyphae transition is favoured by several environmental conditions such as growth at 37^{o}C and near neutral pH or exposure to an inducer like serum, N-acetyl D-glucosamine and proline (284). In the present study, the yeast form of *C.albicans* and AMB-R was converted to hyphae/pseudohyphae using bovine serum or N acetyl D gluocosamine at neutral pH and 37^{o}C. For isolation of cell wall, mechanical disruption using glass beads was the method of choice due to its efficient and complete cell disruption.

Chemical and enzymatic treatment are two methods of isolation of GPI CWPs from hyphal and yeast cells. HF-Pyridine is a method of choice for chemical treatment as it specifically cleaves phosphodiester bonds, through which GPI CWPs are linked to β-1,6-glucan chains. For the enzymatic treatment commercially available β-1,3 glucanase was taken at it cleaves the CWPs which are covalently linked to β-1,3-glucan. The efficiency of these two methods in isolation of GPI CWPs without any contamination of other fraction of cell wall has been reported (25). HF-pyridine efficiently and specifically releases GPI CWPs but does not significantly affect their glycosylation. In the present study in order to identify the immunogenic proteins of GPI origin, the isolated GPI CWPs were immunoblotted with human patient sera and mice polyclonal sera developed against β-1,6-glucan cell wall fraction (Figure 15). A significant difference in the immunogenic protein profile of GPI CWPs from hyphae of both parent and AMB-R strain was observed as compared to yeast form where less number of immunogenic proteins was highlighted. Therefore GPI CWPs from hyphae were used for further work. The bands of immunogenic GPI CWPs of C. *albicans* hyphae were excised from single dimension SDS PAGE gels for MALDI-MS. A total of 7 immunogenic GPI CWPs were identified by peptide mass fingerprinting using the data from mass spectroscopy (Table 2).

Fungal GPI proteins may either be found in the plasma membrane or in the cell wall. Vossen *et al.* (1997) observed that GPI-modified proteins that are predominantly found in the plasma membrane of *S. cerevisiae* generally contain

two basic amino acids upstream of the GPI-anchor attachment site (ω-site). This seems true in case of other fungi as well. Many of the predicted GPI cell wall proteins in the human pathogenic yeasts *C. albicans* and *C. glabrata* are believed to be involved in adhesion and are for that reason presumably exposed to the outer surface of the cell wall (285). Consistently, these proteins generally lack a dibasic motif in the ω-proximal region. Instead, hydrophobic amino acids such as valine, leucine or isoleucine upstream of the ω-site at the ω-2, ω-4, and ω-5 positions seem to act positively to localize the protein to the cell wall. However, the final destination of GPI cell wall proteins is not determined by the ω-proximal region only. Apparently, the presence of long serine- and threonine-rich regions, which are characteristic of many GPI proteins, may favour targeting of a GPI protein to the cell wall and may even override the plasma membrane-retaining effect of a dibasic motif in the ω-proximal region. In the present study, potential GPI CWPs were analyzed for the presence of a GPI-anchor attachment site according to the consensus rules of GPI protein prediction with the help of GPI prediction software PredGPI (http://gpcr.biocomp.unibo.it/predgpi). The prediction of GPI anchor in the identified proteins reemphasizes the specificity of the methods of isolation and validates the identified proteins as GPI CWPs.

Further, all the 7 proteins identified were analysed for their functions *in silico* using *Candida* genome database (CGD). The identified GPI CWPs were subjected to BLASTp software for the identification of function and locus of the protein in *C. albicans*. Many of the predicted fungal GPI CWPs belong to protein family aspartic proteinase and one of them was identified as SAP7 which is known to be expressed during oral and systemic candidiasis (286). One of the other proteins was identified as TDH3, which is a glycolytic enzyme, present on the surface of yeast and hyphae and antigenic in nature during infections. Yet another protein was identified as PTC6 which is a putative protein phosphatase of the Type 2C-related family (serine/threonine-specific). The fourth protein identified was PIR3 which is a putative protein and have a role as structural cell wall component. Among the remaining three proteins one was SSB1 (putative HSP70 family protein and antigenic in human and mice systemic candidiasis) the other one was RIM9 (GPI

protein required for alkaline pH response via the RIM101 signaling pathway) and the last one was KSP1which is a GPI CWP with unknown function.

5.3. Generation of Monoclonal Antibodies

There is an urgent need of “targeted therapy” in the fungal infections due to limited availability, reduced efficacy and side effects of present day antifungal molecules. Targeted therapy refers to a molecule or drug that interveins a specific pathway in pathogen by blocking/attacking an important target. Monoclonal antibody can be used as a molecule for targeted therapy due their specificity to single antigen/epitope and no interaction with other host components. In the era of emergence and wide dissemination of antifungal-resistant strains, especially among immunocompromised hosts, antibody based therapy could represent an effective new weapon in the fight against fungal infections. Antibody-mediated therapy avoids the need for the recipient to be immunocompetent where by not requiring the participation of host adaptive immunity; it hopefully will allow effective treatment (without toxicity) of life-threatening infections in the growing crowd of immunocompromised patients. Furthermore, recent technological advances in the engineering of antibodies and antibody derived peptides may constitute the basis for modelling new and safe receptor-mediated wide-spectrum antimicrobial agents. The current approach of making MAbs against fungal cell surface molecules and then evaluating their efficacy in animal models has revealed numerous antigens that can elicit protective antibody (286). Protective MAbs have been made against classical fungal surface antigens, such as mannans, glucans, and glucuronoxylomannans. Most interestingly, immunization with fungi and fungal lysates has produced unexpected results, identifying antigens that were hitherto not suspected to be targets of antibody-mediated immunity, including surface heat shock and histone-like proteins (287). There is now evidence that proteins, polysaccharides, pigments, and even glycolipids are also targets for protective antibody responses (278).

Thus in the present work, mice were immunized with GPI CWPs in order to generate monoclonal antibodies and to validate GPI CWPs as a new target for antifungal therapy. Longer immunization schedule of 60 days was followed seeking

clones producing the MAbs with stable isotype. Antibody isotype is of immense importance in defence against fungal pathogens and its efficacy to clear fungal infection *in vivo*. In general antibodies of the isotype IgM, IgG1 and IgG3 are known to be protective against *C. albicans* (287). Although monoclonal antibody of IgM isotype is more efficient in clearing antigens and activation of complement system it has some disadvantages. Because of the robust nature of the antibodies of this isotype (IgM is a pentamer) it does not diffuse easily. Also IgM is the first immunoglobulin class produced in a primary response to the antigen by the B cells. The chances of the clones producing IgM isotype undergoing class switching are very high. This is the reason why IgM accounts for only 5-10% of total serum antibodies *in vivo*. Alternatively MAbs of the isotype IgG account for about 80% of the total serum antibodies and are very stable. Therefore most diagnostic techniques are based on IgG isotype. The immunization schedule has tremendous influence on the isotype of the resulting monoclonal antibodies. Longer immunization schedule results in the IgG isotype, while a shorter schedule results in IgM isotype.

In the present study, splenocytes obtained from immunized mice were immortalized by fusion with Sp2/O cell line. The antibody producing hybridoma lines were identified by ELISA and then screened by Western blotting to identify clones that exhibited binding with proteins that were immunogenic in humans. The primary screening after fusion resulted in the identification of 108 clones which were ELISA positive against hyphal GPI CWPs. The number gradually decreased in subsequent screenings and 10 stable clones could be obtained which were consistently producing antibodies. The success rate of positive hybridoma clones is limited and depends on various factors. After fusion, fused cells undergo stress as they are fragile and struggle for the survival for first 10 days post fusion. Further, the ploidy of the fused cells increases (either tetraploid or hexaploid) after fusion and therefore there will be chromosomal rearrangement within first few days and many of these chromosomes are lost due to which cell death, cessation of cell growth and loss of ability to synthesize immunoglobulin takes place resulting in “non-producing” hybrids. In the present study single cell cloning was performed to get rid of these non-producing hybridoma clones. The three stable monoclonal antibody

producing hybridoma clones (2A11, 10D2, and 2C1) were selected for further studies.

Isotyping of the MAbs produced by these clones showed that MAb 2A11 and 10D2 were of the isotype IgG1 and MAb 2C1 was of the isotype IgM. All the three MAbs possessed light chain of the isotype "κ" (Figure 18). Preliminary characterization by Western blotting revealed MAb 2A11 to cross react with two proteins of molecular weight ~46kDa and ~36kDa, MAb 10D2 cross reacted with ~120kDa protein while MAb 2C1 with a protein of ~35 kDa.

5.4. Characterization of MAb2A11, 10D2 and 2C1 for Therapeutic and Diagnostic Potential

The fungal cell wall is a remarkably complex structure that remains poorly understood with regard to its architecture and antigenic composition, yet it is a major target for the immune system. The cell wall is highly immunogenic, and antibodies to cell wall determinants, including β-glucan, are produced during fungal infection. However, prior studies of passive protection with immune sera elicited by fungi produced inconsistent results. This can be explained by the presence of inadequate amounts of protective antibodies or the fact that sera contain both protective and non-protective antibodies. In addition, direct antifungal effects may require a concentration of cell wall-specific antibodies higher than the concentration produced in the course of the immune responses elicited by infection. In the present study, the monoclonal antibodies generated against GPI CWPs were characterized for both therapeutic and diagnosis potential.

The cross reactivity of all the three MAbs 2A11 ,10D2 and 2C1 with the yeast GPI CWPs showed that the antigens were constitutively expressed in the fungus and could be utilized as a marker molecule for the diagnosis of the pathogen. In order to check the diagnostic potential of MAb 2A11, 10D2 and 2C1, cross reactivity studies were carried out with different strains of *C. albicans*. Western blot analysis of 2A11 showed that the antigens were present in all the strains tested. However the upper molecular weight protein (~45kDa) could be detected in very low amounts in two strains of *C. albicans* (Figure 19 A). It is a well known fact that GPI CWPs proteins are transported from cytoplasm and incorporated in to cell wall. The possible

reason for the lower intensity of the upper band could be because of limited incorporation of the protein into cell wall. Cross reactivity studies with different *Candida* species using MAb10D2 also showed that the antigen was constitutively present on the cell wall of the other *C. albicans* strains (Figure 19B). MAb 2A11 and 10D2 did not show any cross reactivity with other yeast form and filamentous forms of fungi indicating their diagnostic potential. MAb 2C1 was also checked for its inter-species and inter-genus specificity (Figure 20). Western blot results indicated that antigen recognised by MAb 2C1 was present in other pathogenic yeast form fungi (*C. parapsilosis*, *C. tropicalis*, *and Cryptococcus neoformans*). The two MAbs along with the MAb 2C1 did not show cross reactivity with pathogenic filamentous fungi (*Aspergillus fumigatus*, *Trichophyton mentagrophytes*, and *Sporothrix schenkii*). Absence of antigen/epitope corresponding to the MAbs tested might have resulted in negative western blotting. The studies clearly indicated that the MAb 2C1 had a broad spectrum capacity of recognising fungal species and may be utilized in the diagnosis of yeast like pathogenic fungi whereas 2A11 and 10D2 can be used in diagnosis of *C. albicans* strains.

C. albicans is a commensal found coexisting with other organisms especially bacteria in normal healthy human beings. In order to check the specificity, cross reactivity studies were carried out against both Gram positive (*Staphylococcus aureus*, *Bacillus cereus*) and Gram negative (*Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruiginosa*) bacteria. All the three MAbs 2A11,10D2 and 2C1 did not exhibit any cross reactivity with any of the bacteria tested indicating its specificity towards fungi.

The cell wall of *C. albicans* is not only the structure that provides the cell with rigidity and protection from the unfavorable environment but it is an essential organelle where many biological functions vital for the survival and growth of this organism reside (220). GPI proteins are the most abundant covalently linked cell wall proteins which are linked to the scaffold of cell wall with β-1,3 glucan and β-1,6 glucan in the elastic three dimensional network of cell wall (221). Majority of these GPI CWPs has been associated with several types of interactions of fungal cells with the extracellular environment (particularly during infection). Therefore, monoclonal antibodies were developed against GPI CWPs of *C. albicans* and

evaluated *in vitro* and *in vivo* for their potential as therapeutic and diagnostic molecule. All the three MAb generated 2A11, 10D2 and 2C1 were also studied for its ability to confer protection *in vitro* against *C. albicans* and AMB-R strain in order to check the effectiveness of the monoclonal antibodies generated against GPI CWPs on amphotericin B resistant strain AMB-R.

To check the specific binding of MAbs with the epitopes of cell wall proteins, the MAbs were used to label intact and fixed *C. albicans* yeast cells and hyphae in indirect immunofluorescence experiments. The epitope localization experiments also included the resistant AMB-R strain in order to observe any changes in epitope. Immunofluorescence microscopy with fluorescein isothiocyanate-conjugated secondary anti-mouse IgGAM revealed that the epitope for 2A11 and 10D2 is uniformly distributed over the entire surface of yeast as well as hyphae in *C. albicans* whereas in AMB-R, the epitope was located on entire surface with higher concentration at basal side in both yeast and in pseudohyphae. The epitopes for MAb 2C1 was distributed in patches over the cell surface in yeast and hyphae of both *C.albicans* and AMB-R (Figure 26). The uniform distribution of epitopes in yeast and hyphae support the presence of proteins in both forms. Majority of GPI CWPs in both yeast and hyphae is located on the cell surface (25). The present results also support the presence of the epitopes on the surface of the cells in both morphological forms. The specific biological activities of the complement system and antibodies that contribute to host resistance are multifaceted and interdependent (289). For example, antibodies contribute to the activation of the complement system by fungi and complement is essential for antibody-mediated protection (290). Complement, collectins, and antibodies promote attachment (opsonisation) and identification of fungi by various receptors (291). In this study, all three MAbs uniformly opsonised the cell surface of *C. albicans* of both yeast and hyphae (Figure. 26).

Adhesion of *C. albicans* to the host tissue is the first step in the infection process. *C. albicans* produces many molecules of biological importance that help in adherence of the pathogen to the host tissue surface in order to penetrate and spread into the host body (292). *C. albicans* exhibits intrinsic property of adhesion to composite material like polyvinyl chloride and poly carbonate plates. Inhibition of

adhesion of *C. albicans* to host surfaces is one of the best-documented activities mediated by antibodies, and different degrees of inhibition have been described with saliva, polyclonal antisera, and MAbs (293). The inhibition of adhesion is usually mediated by blocking the adhesins present on the fungal cell wall. MAbs 2A11 and 10D2 showed significant inhibition in adherence of *C. albicans* to the surface of the poly carbonate plate in both parent *C. albicans* and AMB-R whereas 2C1 showed the minimum inhibition (Figure 22).

The inhibition of adherence can be correlated well with the amount of antigen present on the surface of cell wall (Figure 26). The immuno fluorescence results revealed that the maximum fluorescence was obtained for MAb 2A11 and MAb 10D2 followed by MAb 2C1. In addition, the amount of fluorescence on cells incubated with MAb 2A11 and MAb 10D2 was evenly distributed and spread over the entire surface of yeast as well as hyphae while in case of MAb 2C1 it was uniformly distributed with lighter patches present in certain regions of the cells. Thus it could be concluded that the antibodies that bind with the surface antigens inhibit attachment of fungal cells with the host tissue surface and the extent of inhibition is decided by distribution pattern and concentration of the epitope on cell envelop (294). These findings are in agreement with the observation of other researchers who reported characterization of three monoclonal antibodies against cell surface proteins of *C. albicans*, and found that the antibodies were instrumental in inhibition of attachment with extracellular matrix proteins (290, 295). Han *et al* (290) found that one of their monoclonal antibody had a patchy distribution that failed to protect mice against a systemic challenge while the one that had an even distribution was effective in prevention of candidiasis. These observations suggest that monoclonal antibodies described in this work have the potential to protect animal models of *Candida* infection.

The conversion of yeast to hyphae is an interesting phenomenon exhibited by *C. albicans* that is believed to play an important role in pathogenesis of candidiasis (8). This morphological transition is regulated by a set of genes that results in an increased expression of adhesins on the surface of hyphal wall that are required for attachment with host tissue. Mutations leading to defects in hyphae formation are associated with a reduction or complete abrogation of virulence in these mutated

strains. Monoclonal and polyclonal antibodies specific for cell surface epitopes have been shown to inhibit yeast-hyphal morphogenetic transformation (294). Such antibodies were found to offer protection in animal models of candidiasis. The monoclonal antibodies described in the present study were also evaluated for their ability to inhibit germ tube formation. Monoclonal antibody 10D2 was found to be the best as it reduced the germ tube formation to 75% whereas Mab2C1 showed the least (19%) inhibition. The effect of MAbs on inhibition of germination on the resistant strain AMB-R could not be explained as this strain has intrinsic reduced germ tube formation capacity where there was no difference in the germ tune formation in the presence of monoclonal antibodies as compared to control.

The need for adding novel, in particular immunotherapeutic weapons to our antimicrobials armamentarium is largely recognized partly due to emergence of resistance to known antimicrobial agents and partly due to paucity of new antimicrobial with a completely different mode of action. In the present work, the candidacidal potential of monoclonal antibodies was evaluated initially *in vitro* to assess the cidal activity of monoclonal antibodies (Figure 21). The monoclonal antibodies 2A11 and 10D2 exhibited nearly 90% reduction in cfu whereas MAb2C1 did not exhibit significant reduction in cfu (39%). Since this reduction in cfu may also be due to agglutination caused by monoclonal antibodies, MTT assay was performed (Figure 24). This assay evaluated the growth in terms of ability of live cells to metabolize the MTT-dye. An inhibition of 91% and 89% was observed in case of MAb 2A11 and 10D2 whereas gowth inhibition due to MAb 2C1 was only 25% (Figure 24). Both the above methods suffer from a drawback that the incubation period involved may shift the metabolism of *C. albicans* from an exponentially growing cell to that of a latent and resting blastospore. This may be one of the possibilities that a very high growth inhibition was observed in the MTT and *in vitro* activity assay described in this work.

Thus in order to rule out these drawbacks, flow cytometry was used to evaluate of cidal activity on the basis of differential staining and counting of live and dead cells. Flow cytometry provides simple and high throughput system to evaluate antifungal activity and is applicable for molecules as diverse as monoclonal antibodies. In this method the cells of *C. albicans* were exposed to the effective concentrations (*in*

vitro activity and MTT assay) of MAb 2A11 and MAb 10D2 for 1h, and the live and dead cells were differentially stained with PI (stains dead cells) and FDA (stains live cells) and kept on ice till they were analyzed on flow cytometer (Figure 25). As MAb 2C1 did not show any *in vitro* cidal activity it was excluded from the flow cytometry analysis. Both the MAbs 2A11 and 10D2 did not exhibit much variation in their cidal activities and the results were in accordance to that seen under MTT and *in vitro* assay. It rules out any possibilities of killing of cells due to longer incubation or any other environmental factors. In this analysis, AMB was taken as positive control where over 90% killing of parent *C. albicans* cells was observed, which is in accordance with the published data (296) for this antifungal as determined by other methods whereas AMB-R showed minimal killing in presence of amphotericin B. The flow cytometry also exhibited the presence of MAb 2A11 and 10D2 exerts cidal activity on resistant strain AMB-R which was lower than found against its parent *C. albicans*. The killing of drug resistant strain in the presence of monoclonal antibodies emphasizes its role in the treatment of drug resistance cases due to their specificity for target molecule irrespective of mutation in the resistant cases.

There is an increasing interest in novel, immune-based prophylactic and therapeutic approaches to treat invasive candidiasis. Cell-mediated immunity and innate immunity are considered to be the most important lines of defense against candidiasis. Recently it has been demonstrated that antibodies with defined specificities show different degrees of protection against systemic and mucosal candidiasis (149, 168). However, the perspectives of effective immunotherapy against fungal infections have so far been very limited (28). It is quite logical that the availability of protective antibodies targeting common, viability-critical components of fungal cells may greatly expand the potential and clinical perspectives for antifungal immunotherapy. Cell wall or components of cell wall like β-Glucan is one such target that is present in all human pathogenic fungi, although it has distinctive molecular features in the various fungus genera (201). Following this strategic line, anti β-glucan antibodies produced in mouse recipients of a glucan-conjugated vaccine have recently been shown to confer protection against *C. albicans* as well as *A. fumigatus* infections (201, 287). In particular, an IgG2b anti- β-glucan MAb has been generated that has shown *in vitro* and *in vivo*

protective activity. This MAb was shown to efficiently bind hyphae of both fungi and germinating *Aspergillus* conidia, thus inhibiting hyphal growth *in vitro* (41). In the present study the MAbs generated against GPI CWPs were evaluated for their therapeutic value in murine model of Candidiasis. The basic method for evaluating the efficacy of antibody-mediated immunity involves the administration of specific antibody to a host followed by microbial challenge. Here also, the mice were challenged with *C. albicans* after MAb 2A11 and 10D2 administrations and the protection was evaluated on the basis of number of cfu in the kidney tissues of control and treated mice (Figure 28). Both the antibodies conferred protection against candidiasis as was demonstrated by reduction in cfu counts in kidney tissues. These results are comparable to those reported by other groups using different anti-*C. albicans* MAbs (180).

Antibodies exhibit numerous responsibilities such as neutralization of the toxins secreted by the pathogens, activation of the complement, participation in antibody dependent cell cytotoxicity, opsonisation of the pathogens along with the activated complement under *in vivo* conditions. MAb 2C1 was evaluated for its ability to enhance phagocytosis of opsonised *C. albicans* yeast cells and germ tubes by mouse macrophage cell line. The process of phagocytosis could be seen within an hour of co-culturing of the MAb treated and PBS treated cells with the macrophages. Marked enhancement of phagocytosis could be noticed in case of MAb 2C1 treated cells as compared to that of PBS treated cells (Figure 27). Thus MAb 2C1 contributed to the protection against *C. albicans* in an indirect way by enhancing phagocytosis. The study also indicated that the antigens recognized by the MAb 2C1 were present on the surface of the pathogen and were not hidden in the complex structure of the cell wall.

Antibodies (Abs) are formed by heavy and light chains composed of constant and variable regions. The variable light chain region includes three complementarity determining regions (CDRs) which constitute the antigen (Ag) binding-site. The observation that Ab specificity is determined by a limited number of residues has prompted the synthesis of small peptides based on CDR sequences which retain binding properties and functions of the intact Ab (296, 297). In previous studies it has been demonstrated that the CDRs, or related peptidic fragments, of a

recombinant single chain Ab (scFv), representing the internal image of a wide antimicrobial spectrum Pichia anomala killer toxin (KT), may exert a specific microbicidal activity *in vitro* against KT-sensitive microorganisms characterized by specific cell-wall receptors mainly constituted by 1,3-β-glucans (298). One of its derivatives (KP) characterized by a significant increase of the candidacidal activity was able to exert a very effective therapeutic activity in experimental models of vaginal and systemic candidiasis, disseminated cryptococcosis and paracoccidioidomycosis. Polonelli et al. (187) studied synthetic peptides with sequences identical to CDRs of the light and heavy chain of three monoclonal Abs (MAbs) characterized by different specificity: MAb C7, directed to a protein epitope of a *Candida albicans* (*C. albicans*) cell wall stress mannoprotein, mAb pc42, directed to a synthetic peptide containing well-characterized B-cell and T-cell epitopes, and a human MAb HuA, directed to blood group A substance (180, 299, 300, 301). The study showed that, irrespective of the specificity of the native Ab, the synthetic CDRs may exert *in vitro*, *ex vivo* and/or *in vivo* differential inhibitory activities against *C. albicans*, HIV-1 and B16F10-Nex2 melanoma cells, conceivably mediated by different mechanisms of action. As bioactive molecules, CDR-related peptides may present some advantages over whole Abs of adaptive immunity owing to their small size, e.g. lack of immunogenicity and better tissue penetration, as well as over natural peptides of innate immunity (e.g. defensins, cathelicidins, hystatins) owing to higher specificity and affinity for targets and low systemic toxicity (302, 303). In the present study, the CDRs of MAb 2A11 were determined and peptides were synthesized. CDR sequences in the variable regions of immunoglobulins are thought to act cooperatively in the recognition of an antigen. The synthesized peptide did not show any significant activity *in vitro* in both the strains parent *C. albicans* and AMB-R. The observation that Ab specificity is determined by a limited number of residues has allowed the synthesis of small peptides based on CDRs which retain binding properties and functions of the intact Ab could be the possible reason of lesser *in vitro* activity of peptides as compare to intact monoclonal antibody.

5.5. Conclusion

In the present study, amphotericin B resistant strain (AMB-R) was developed and characterized under labroratory conditions in order to unreveal the drug resistance mechanism. The stability of resistance was confirmed *in vivo* and with time kill curves. The resistant strain AMB-R was compared to its parent strain at morphological, biochemical, physiological and genetic level. The various changes had been observed in AMB-R strain which may be either reasons for amphotericin B resistance or resultants of drug exposure. The morphology of AMB-R strain had been changed to pseudohyphae from hyphae. The germ tube formation capacity and glucan synthase activity was also found to be reduced in AMB-R strain. The major component of cell membrane ergosterol was also found to be reduced and the expression level of one of the ergosterol biosynthetic pathways gene ERG11 was studied at nucleotide and expression level. The expression of ERG11 was increased in the case of AMB-R strain as compared to parent strain. The changes at nucleotide level were also observed in ERG11 and CSP37. Virulence factors like secreted aspartyl proteinase and phospholipase had increased activity in AMB-R strain.

Covalently linked cell wall proteins were isolated from both parent and AMB-R strain. Hyphae GPI CWPs of parent *C. albicans* strain was used in identification of immunogenic proteins on the basis of their higher reactivity towards patient and polyclonal sera. Seven GPI CWPs were identified using peptide mass fingerprinting and GPI anchor prediction. Further, GPI CWPs were used to generate hybridoma clones producing monoclonal antibodies against these proteins with therapeutic or diagnostic potential. Three stable hybridoma clones designated MAb 2A11, MAb 10D2 and MAb 2C1 (IgG1, IgG1 and IgM isotype respectively) were obtained from the fusion experiment. Western blot analysis showed that the proteins recognized by MAb 2A11 and 10D2 is a constitutively expressed proteins and are present only in all *Candida albicans* strains tested including AMB-R whereas 2C1 showed cross reactivity with proteins of all the yeast form fungi but not in the pathogenic filamentous fungi. Thus it has a broad pathogenic spectrum and could be effectively utilized for diagnostic purpose. The monoclonal MAb 2C1 exhibited diagnostic potential (51%) in *in vivo* experiments which could be enhanced by increasing the

population size. The MAb 2C1 exhibited a good potential during *in vitro* screening (56.8%) of *Candida* positive patient sera for the presence of antigens recognized by the MAb. The MAb 2C1 increased phagocytosis of both parent and AMB-R strain by mouse macrophage J774.The MAbs 2A11 and 10D2 exhibited *in vitro* activity against both parent and AMB-R strain. All the three MAbs were subjected different biological anti-*Candida albicans* activities like to inhibition to adhesion, MTT assay, Flow cytometry, inhibition of germination. Only 2A11 and 10D2 showed therapeutic potential against parent *C. albicans* and AMB-R strain indicating the possibility of treatment of drug resistance cases using monoclonal antibody based therapy. Both the antibody showed protection against murine model of candidiasis. The peptides were designed and synthesized on the basis of CDRs sequences of light chain of MAb2A11.The eight peptides were subjected for in vitro activity in parent *C. albicans* and AMB-R. Only two peptides showed *in vitro* activity against both the strain tested. Since the monoclonal antibodies generated against GPI CWPs in the present study have shown their potential for diagnosis and experimental therapy it may be concluded that such studies may lead to find more effective tools for diagnosis and therapy of fungal infections. Covalently linked cell wall proteins, GPI CWPs in particular, may be used as target molecules to develop safe and precise monoclonal antibody based antifungal therapy.

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Appendix

Table 1: Enzymatic activities of parent *C. albicans* and AMB-R in H_2O_2 yeast form

Enzymes	Catalase		GPx		GST	
Treatment	ATCC10231	AMB-R	ATCC10231	AMB-R	ATCC10231	AMB-R
Control	86.29 ±4.64	166.48± 7.96	49.20±9.97	90.77±18.42	83.75±22.99	169.19±20.86
5mM H_2O_2	81.16 ± 7.39	154.31± 14.18	57.15±9.20	95.435± 8.26	35.41±8.52	126.98±46.6
50mM H_2O_2	43.85 ± 9.24	120.86± 25.80	31.89±6.83	75.004±12.59	22.94±3.50	82.48±8.49
5+50mM H_2O_2	78.36 ±9.74	139.46± 16.27	56.00±12.0	111.99±19.77	33.00±6.93	94.40±8.92

Hyphae/Pseudohyphae

Enzymes	Catalase		GPx		GST	
Treatment	ATCC10231	AMB-R	ATCC10231	AMB-R	ATCC10231	AMB-R
Control	69.040±7.32	134.063±15.9	46.44 ± 6.65	129.72±22.34	79.65±12.7	116.80±2.3
5mM H_2O_2	70.106±7.77	101.440±14.7	42.26 11.05	111.19±20.86	66.80±10.5	85.13±12.5
50mM H_2O_2	44.866±6.60	99.950±28.05	32.85 ±6.99	65.32±10.46	41.85±5.28	65.68±13.46
5+50mM H_2O_2	64.923± 8.93	118.276±15.1	34.75 ±7.53	65.87±6.03	55.50±10.8	80.53±6.64

Table2: Enzymatic activities of parent *C. albicans* and AMB-R in menadione yeast form

Enzymes	Catalase		GPx		GST	
Treatment	ATCC10231	AMB-R	ATCC10231	AMB-R	ATCC10231	AMB-R
Control	86.29±4.64	166.43±7.96	49.20±9.97	90.77±18.	83.75±22.9	169.19±20.8
0.2mM	80.04±3.40	144.4± 10.03	46.79±5.83	88.64±5.5	71.39±22.2	179.83±29.0
0.8mM	75.06±8.72	99.03± 18.90	37.24±5.93	85.54±7.0	27.72±14.3	120.24±18.4
1.6mM	33.22±11.40	94.79±7.11	29.95±2.75	62.88±9.4	26.06±11.6	104.11±25.6
3.2mM	15.32±7.51	85.99±10.10	18.37±3.19	47.43±13.	21.51±9.86	75.89±20.37

hyphae/pseudohyphae

Enzymes	Catalase		GPx		GST	
Treatment	ATCC10231	AMB-R	ATCC10231	AMB-R	ATCC10231	AMB-R
Control	69.04 ± 7.3	134.06± 15.93	46.44±6.65	129.72±22.3	79.65±12.7	116.80±23.49
0.2mM	86.39 ± 9.60	145.80 ± 9.93	32.47±5.86	88.24±11.04	65.94±7.54	87.31±11.67
0.8mM	36.26 ± 5.52	87.73 ± 4.99	31.55±12.42	73.08±14.29	45.83±7.55	79.81±5.66
1.6mM	27.56 ± 5.17	85.52 ± 8.03	24.71±9.34	47.51±13.53	42.47± 6.45	72.72±6.44
3.2mM	22.00 ± 5.41	61.90 ± 13.96	22.85±7.07	44.16±10.28	40.87± 8.48	68.15±8.44

Peptide mass fingerprinting Score:

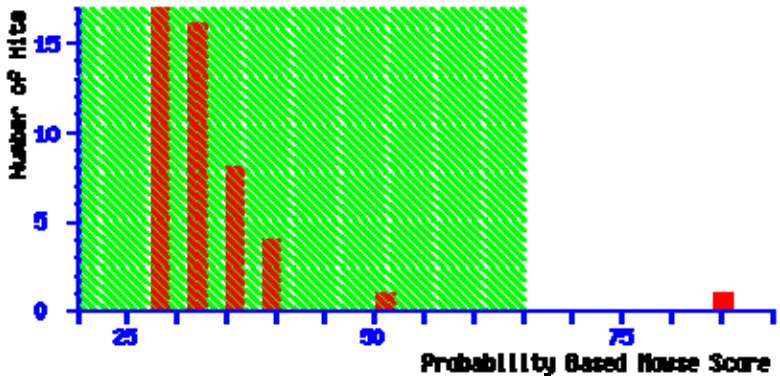
I. Proteins identified through MASCOT

(A).TDH3

Top Score: 85 for Q5ADM7_CANAL,Glyceraldehyde-3-phosphate dehydrogenase *C. albicans* (Yeast).

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 65 are significant (p<0.05).



Match to: **Q5ADM7_CANAL** Score: **85** Expect: **0.00056**

Glyceraldehyde-3-phosphate dehydrogenase.- Candida albicans (Yeast).

Nominal mass (M_r): **35811**; Calculated pI value: **6.61**. Matched peptides shown in **Bold**

1 M**AIKIGINGF GR**IGRLVLRV ALGRKDIEVV AVNDPFIAPD YAAYMFK**YDS**

51 **THGRYKGEVT ASGDDLVIDG HK**IK**VFQERD PANIPWGKSG VDYVIESTGV**

101 **FTK**LEGAQKH IDAGAKKVII TAPSADAPMF VVGVNEDKYT PDLKIISNAS

151 CTTNCLAPLA K**VVNDTFGIE EGLMTTVHSI TATQK**TVDGP SHKDWRGGRT

201 ASGNIIPSST GAAKAVGKVI PELNGKLTGM SLR**VPTTDVS VVDLTVR**LKK

251 **AASYEEIAQA IKK**ASEGPLK **GVLGYTEDAV VSTDFLGSSY SSIFDEK**AGI

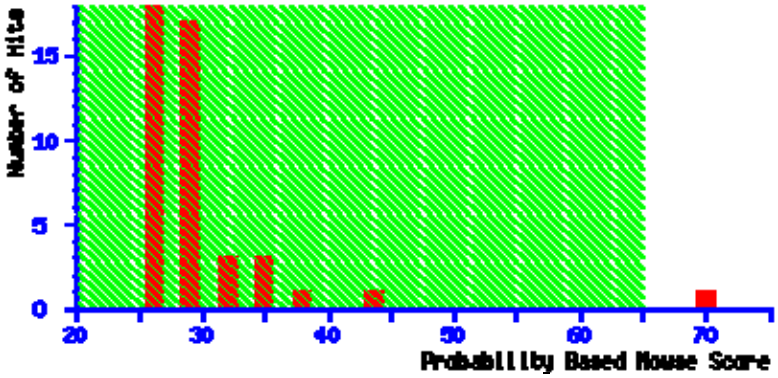
301 LLSPTFVK**LI SWYDNEYGYS TRVVDLLEHV AK**ASA

(B).SAP7

Top Score : 70 for S42074, aspartic proteinase (EC 3.4.23.-) SAP7 - yeast *C. albicans*

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 65 are significant (p<0.05).



Match to: **Q6BMN8_DEBHA** Score: **46** Expect: **4.7** Nominal mass (M_r): **49899**; Calculated pI value: **9.11** Matched peptides shown in **Bold**

1 MGKEKTHVNL VVIGHVDSGK STTTGHLIYK **CGGIDKR**TIE K**FEKEAAELG**

51 KGSFKYAWVL DKLKAER**ER**G ITIDIALWKF ETPKFHVTII DAPGHRDFIK

101 NMITGTSQAD CAILIIAGGI GEFEAGISKD GQTREHALLA YTLGVKQLIV

151 AINKMDSVKW DKNRYDEIVK ECSNFVKKVG FNPK**SVPFVP ISGWNGDNMI**

201 EASPNCPWYK GWEKETKAGK **SSGKTLLEAI DAIEPPSRPT DKPLRLPLQD**

251 VYKIGGIGTV PVGRVETGII KAGMVVTFAP AGVTTEVKSV EMHHEQLTEG

301 VPGDNVGFNV KNVSVKEIRR GNVCGDSK**ND TPKGCDSFAA QVIVLNHPGQ**

351 ISSGYSPVLD CHTAHIACKF DTLIEKIDRR TGK**KLEDNPK** FIKSGDAAIV

401 KMVPSKPMCV EAFTDYPPLG RFAVRDMRQT VAVGVIKSVE KSDKAGKVTK

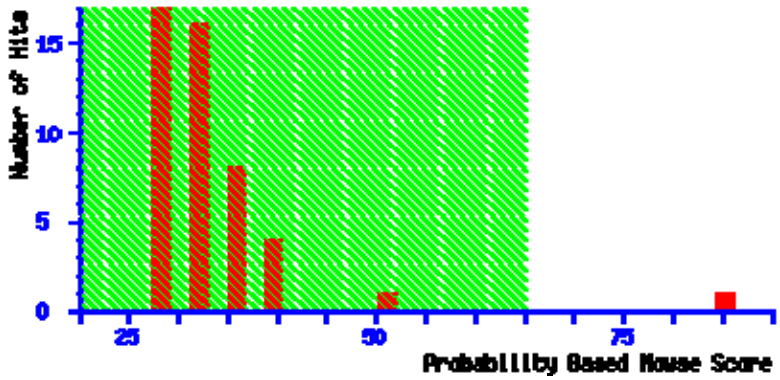
451 AAQKAAKK

(C)PTC 4

Top Score : 85 for Q6BNG6_DEBHA, Similar to CA4082|CaPTC4 *C. albicans* CaPTC4 ser/thr protein phosphatase PP2C.

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 65 are significant (p<0.05).



Protein View

atch to: **Q6BNG6_DEBHA** Score: **41** Expect: **13**

Nominal mass (M_r): **38173**; Calculated pI value: **5.61**

Matched peptides shown in **Bold**

1 M**GQLLSHPIE DKTMEHK**SYD TITYCIGLMQ GYRMSMEDAH NVKVNEDESL

51 AVFGVFDGHG GKTCAEVVSD KLPTMVFREL SSLLKNGNGD LASYMKVLKD

101 SFFR**IDRDLT NEDSSNCGTT AIIASIIANE YIIVSNAGDS RCIMSLEGGA**

151 PKTLSFDHKP STMGERVRIE NSGGYVVNGR VNEILALSRA FGDFKFKLPY

201 MELLNNQNKY IAANKK**YFKH ELIHLPPELF LVSVEPDVVV YDLK**SLKQPE

251 FVVLACDGIW DCYTNTKLIK IIRDKLSLDW KIHHITEFIL NDCVGMANNV

301 TGIGFDNMTI IIVAVHNNNN IDEWYTMMK**E R**VLKEKGLL

II. Proteins Identified using Aldente and pepMAPPER

(D). TOR1

Protein_Hit: P35169 *TOR1_YEAST Phosphatidylinositol 3-kinase TOR1 (EC 2.7.1.137) (PI3-kinase) (PtdIns-3-kinase) (PI3K)*
Score: 2.978e-01 Matches: 8.1% *Len: 2470 Mass: 280942.3 pI: 9.00 coverage: 1.4% overlap: 755696.2%*

Search Pep	Search M/Z	Database M/Z	Database mass	Mass diff	Peptide Start	Peptide Length	Z	P	Sequence
1	304.150	304.154	303.154	0.00	538	2	1	0	ER
22	2590.420	2590.326	2589.326	0.09	672	23	1	0	LLFTALHDESFNIQSVAMELVGR
1	304.150	304.154	303.154	0.00	874	2	1	0	ER
7	842.290	842.328	841.328	0.04	2148	7	1	0	NDSECFK

(E). RIM9

Protein_Hit: Q04734 *RIM9_YEAST RIM9 protein*
Score: 5.792e-03 Matches: 3.2% *Len: 239 Mass: 26678.7 pI: 9.13 coverage: 4.2% overlap: 4227099.6%*

Search Pep	Search M/Z	Database M/Z	Database mass	Mass diff	Peptide Start	Peptide Length	Z	P	Sequence
8	1152.330	1152.330	1151.330	0.00	51	10	1	0	GQNQELMCTK

(F).SSB2

Protein_Hit: P40150 *HS76_YEAST Heat shock protein SSB2*
Score: 1.050e-01 Matches: 10.5% *Len: 612 Mass: 66404.8 pI: 5.77 coverage: 4.7% overlap: 1925887.6%*

Search Pep	Search M/Z	Database M/Z	Database mass	Mass diff	Peptide Start	Peptide Length	Z	P	Sequence
1	304.150	304.154	303.154	0.00	194	2	1	0	ER
10	2807.530	2807.566	2806.566	0.04	393	27	1	0	DLLLLDVAPLSLGVGMQGDIFGIVVPR

(G).PIR3

Protein_Hit: Q03180 *PIR3_YEAST PIR3 protein precursor (Covalently-linked cell wall protein 8)*
Score: 1.624e-02 Matches: 3.2% *Len: 361 Mass: 36336.1 pI: 9.22 coverage: 19.9% overlap: 3291279.8%*

Search Pep	Search M/Z	Database M/Z	Database mass	Mass diff	Peptide Start	Peptide Length	Z	P	Sequence
23	1745.900	1745.896	1744.896	0.00	90	18	1	0	STAAAVSQITDGQVQAAK
23	1745.900	1745.896	1744.896	0.00	126	18	1	0	STAAAVSQITDGQVQAAK
23	1745.900	1745.896	1744.896	0.00	144	18	1	0	STAAAVSQITDGQVQAAK
23	1745.900	1745.896	1744.896	0.00	180	18	1	0	STAAAVSQITDGQVQAAK

Statistical Analysis using Graph Pad Prism Software:

1. Effect of Monoclonal Antibodies on Colony Forming Units

Bonferroni posttests

DPBS vs 2A11

Column Factor	DPBS	2A11	Difference	95% CI of diff.
ATCC10231	4.333	99	94.67	80.37 to 101.0
AMB-R	6.333	86.67		79.33 to 89.63

Column Factor	Difference	t	P value	Summary
ATCC10231	90.67	26.81	P<0.001	***
AMB-R	79.33	23.54	P<0.001	***

DPBS vs 10D2

Column Factor	DPBS	10D2	Difference	95% CI of diff.
ATCC10231	4.333	93.33		88 78.71 to 99.29
AMB-R	6.333	85		78.67 68.37 to 88.96

Column Factor	Difference	t	P value	Summary
ATCC10231	89	26.41	P<0.001	***
AMB-R	78.67	23.34	P<0.001	***

DPBS vs 2C1

Column Factor	DPBS	2C1	Difference	95% CI of diff.
ATCC10231	4.333	34.67		30.33 20.34 to 40.33
AMB-R	6.333	30.33		24 13.71 to 34.29

Column Factor	Difference	t	P value	Summary
ATCC10231	30.33	9.002	P<0.001	***
AMB-R	24	7.122	P<0.001	***

DPBS vs Irrivalent MAb

Column Factor	DPBS	Irrivalent MAb	Difference	95% CI of diff.
ATCC10231	4.333	15.33		11 0.7352 to 21.29
AMB-R	6.333	15		8.667 -1.628 to 18.96

Column Factor	Difference	t	P value	Summary
ATCC10231	11	3.264	P<0.01	**
AMB-R	8.667	2.572	P < 0.05	*

2. Inhibition of binding to surface

Bonferroni posttests

DPBS vs 2A11

Column Factor	DPBS	2A11	Difference	95% CI of diff.
ATCC10231	183.7	125	58.67	-125 -157.4 to -92.59
AMB-R	172.7	101.3	71.33	-101.3 -133.7 to -68.93

Column Factor	Difference	t	P value	Summary
ATCC10231	-125	11.78	P<0.001	***
AMB-R	-101.3	9.552	P<0.001	***

DPBS vs 10D2

Column Factor	DPBS	10D2	Difference	95% CI of diff.
ATCC10231	183.7	147	37	-146.7 -179.1 to -114.3
AMB-R	172.7	135	37.67	-135 -167.4 to -102.6

Column Factor	Difference	t	P value	Summary
ATCC10231	-146.7	13.83	P<0.001	***
AMB-R	-135	12.73	P<0.001	***

DPBS vs 2C1

Column Factor	DPBS	2C1	Difference	95% CI of diff.
ATCC10231	183.7	60.33	123.3	-60.33 -92.74 to -27.93
AMB-R	172.7	52.33	120.3	-52.33 -84.74 to -19.93

Column Factor	Difference	t	P value	Summary
ATCC10231	-60.33	5.667	P<0.001	***
AMB-R	-52.33	4.933	P<0.001	***

DPBS vs Irrl MAb

Column Factor	DPBS	Irrl MAb	Difference	95% CI of diff.
ATCC10231	183.7	8.333	175.3	-8.333 -40.74 to 24.07
AMB-R	172.7	166.7	178.7	6 -26.41 to 38.41

Column Factor	Difference	t	P value	Summary
ATCC10231	-8.333	0.7656	P > 0.05	ns
AMB-R	6	0.5656	P > 0.05	ns

3. Inhibition of germination of *Candida* cells

Bonferroni posttests

DPBS vs 2A11

Column Factor	DPBS	2A11	Difference	95% CI of diff.
ATCC10231		8	45	37 to 24.61 to 49.39
AMB-R		8.333	22	13.67 to 1.278 to 26.06

Column Factor	Difference	t	P value	Summary
ATCC10231		37	9.124 P<0.001	***
AMB-R		13.67	3.37 P<0.01	**

DPBS vs 10D2

Column Factor	DPBS	10D2	Difference	95% CI of diff.
ATCC10231		8	66	58 to 45.61 to 70.39
AMB-R		8.333	26	17.67 to 5.278 to 30.06

Column Factor	Difference	t	P value	Summary
ATCC10231		58	14.3 P<0.001	***
AMB-R		17.67	4.357 P<0.001	***

DPBS vs 2C1

Column Factor	DPBS	2C1	Difference	95% CI of diff.
ATCC10231		8	24	16 to 3.611 to 28.39
AMB-R		8.333	22	13.67 to 1.278 to 26.06

Column Factor	Difference	t	P value	Summary
ATCC10231		16	3.946 P<0.01	**
AMB-R		13.67	3.37 P<0.01	**

DPBS vs Irrivalent MAb

Column Factor	DPBS	Irrivalent MAb	Difference	95% CI of diff.
ATCC10231		8	8	0 to -12.39 to 12.39
AMB-R		8.333	6.333	-2 to -14.39 to 10.39

Column Factor	Difference	t	P value	Summary
ATCC10231		0	0 P > 0.05	ns
AMB-R		-2	0.4932 P > 0.05	ns

4. MTT assay

Bonferroni posttests

DPBS vs 2A11

Column Factor	DPBS	2A11	Difference	95% CI of diff.
ATCC10231		2.333	89.67	87.33 to 77.10 to 97.57
AMB-R		5.667	88.07	82.47 to 72.17 to 92.83

Column Factor	Difference	t	P value	Summary
ATCC10231		87.33	26.07 P<0.001	***
AMB-R		82.4	24.6 P<0.001	***

DPBS vs 10D2

Column Factor	DPBS	10D2	Difference	95% CI of diff.
ATCC10231		2.333	86	83.67 to 73.43 to 93.90
AMB-R		5.667	89.67	84 to 53.77 to 74.23

Column Factor	Difference	t	P value	Summary
ATCC10231		83.67	24.98 P<0.001	***
AMB-R		84	19.11 P<0.001	***

DPBS vs 2C1

Column Factor	DPBS	2C1	Difference	95% CI of diff.
ATCC10231		2.333	34.33	32 to 21.77 to 42.23
AMB-R		5.667	26.67	21 to 10.77 to 31.23

Column Factor	Difference	t	P value	Summary
ATCC10231		32	9.554 P<0.001	***
AMB-R		21	6.27 P<0.001	***

DPBS vs Irrivalent MAb

Column Factor	DPBS	Irrivalent MAb	Difference	95% CI of diff.
ATCC10231		2.333	14.67	12.33 to 2.101 to 22.57
AMB-R		5.667	13.33	7.667 to -2.566 to 17.90

Column Factor	Difference	t	P value	Summary
ATCC10231		12.33	3.682 P<0.01	**
AMB-R		7.667	2.289 P > 0.05	ns

Research Publications

1. **Rohitashw Kumar** and P.K Shukla. Role of antioxidant enzymes in Amphotericin B resistance and oxidative stress tolerance in *Candida albicans* (MS under preparation)
2. **Rohitashw Kumar** and P.K Shukla..Monoclonal antibody generated against GPI cell wall proteins has candida cidal activity. (MS under preparation).
3. **Rohitashw Kumar**, N.N Mishra and P. K. Shukla Arachidonic acid, fluconazole and terbinafine affect biofilm formation and PGE level in *albicans* and non-*albicans Candida* species. IJAA, 2009. (communicated)
4. **Rohitashw Kumar** and P.K Shukla, Amphotericin B resistance leads to enhanced Proteinase and phospholipase activity and reduced germ tube formation in *Candida albicans*. **Mycological Research 2009, Accepted**
5. Chaturvedi AK, **Kumar Rohitashw** , Kumar A, Shukla PK. A monoclonal IgM directed against immunodominant catalase B of cell wall of Aspergillus fumigatus exerts anti-A. fumigatus activities. **Mycoses 2008,** 52, 524–533