



Analysis of human host defense mechanisms against the opportunistic pathogen, *Candida albicans*, using in vitro model systems
by Heather Amy Edens

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University
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Abstract:

Various aspects of neutrophil-*Candida* interactions were investigated. Serum-free culture filtrates of five *Candida* species and *Saccharomyces cerevisiae* were found to contain chemoattractants for human neutrophils and a mouse macrophage-like cell line, J774. The chemoattractant in *Candida albicans* culture filtrates appeared to act through the formyl peptide receptor (FPR) of neutrophils, since it was also found to induce chemotaxis of Chinese hamster ovary (CHO) cells expressing the human FPR, but not wild type CHO cells. Therefore, we have identified a receptor by which a non-serum dependent chemotactic factor (NSCF) produced by *C. albicans* induces chemotaxis of neutrophils. J774 cells did not migrate toward the formylated peptides and chemotaxis toward the *C. albicans* culture filtrate was not inhibited by an FPR antagonist, suggesting that a different receptor mediated J774 cell chemotaxis. The *C. albicans* culture filtrates also induced neutrophil migration across monolayers of a human gastrointestinal (GI) epithelial cell line, T84, in the basolateral-to-apical direction, but not the reverse, unless epithelial tight junctions were disrupted. This observation suggested that NSCFs produced by *C. albicans* and other yeast species may influence host-pathogen interactions at the GI mucosa by inducing phagocytic cell infiltration.

The role of β 2-integrins in phagocytosis and killing of *C. albicans* was confirmed using neutrophils isolated from calves with bovine leukocyte adhesion deficiency (BLAD). BLAD neutrophils displayed decreased phagocytosis and killing capabilities, supporting claims that β 2-integrins are the primary PMN adhesins for *C. albicans*.

To examine the differences in adhesion of *Candida* species to human GI epithelium, radiolabeled yeast were allowed to adhere to T84 and Caco2 monolayers. The adhesion to T84 monolayers correlated with the reported prevalence of *Candida* spp. in the human GI tract and the adhesion to Caco2 monolayers was similar, although *C. glabrata* displayed greater adherence than other yeast tested. *C. tropicalis* adhered poorly to both cell lines in contrast to exfoliated buccal and vaginal epithelial cells, suggesting differences in expression of cell adhesion molecules on different epithelial cell types and/or *Candida* species. Therefore, human intestinal epithelial cell lines are a useful in vitro model system to investigate the initial steps of colonization of the human GI tract.

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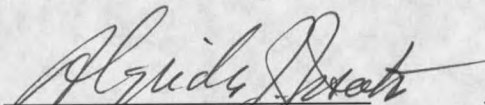
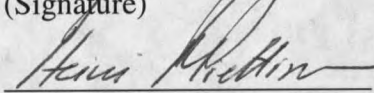
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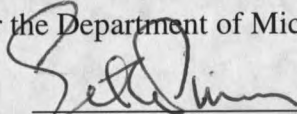
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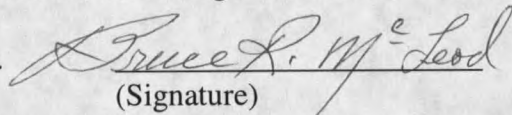
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	(Signature)	Date
Dr. Heini M. Miettinen, Ph.D.		<u>8/24/99</u>
	(Signature)	Date

Approved for the Department of Microbiology

Dr. Seth H. Pincus, MD		<u>8/24/99</u>
	(Signature)	Date

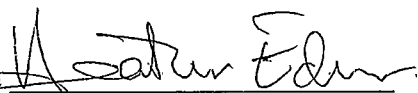
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ABSTRACT

Various aspects of neutrophil-*Candida* interactions were investigated. Serum-free culture filtrates of five *Candida* species and *Saccharomyces cerevisiae* were found to contain chemoattractants for human neutrophils and a mouse macrophage-like cell line, J774. The chemoattractant in *Candida albicans* culture filtrates appeared to act through the formyl peptide receptor (FPR) of neutrophils, since it was also found to induce chemotaxis of Chinese hamster ovary (CHO) cells expressing the human FPR, but not wild type CHO cells. Therefore, we have identified a receptor by which a non-serum dependent chemotactic factor (NSCF) produced by *C. albicans* induces chemotaxis of neutrophils. J774 cells did not migrate toward the formylated peptides and chemotaxis toward the *C. albicans* culture filtrate was not inhibited by an FPR antagonist, suggesting that a different receptor mediated J774 cell chemotaxis. The *C. albicans* culture filtrates also induced neutrophil migration across monolayers of a human gastrointestinal (GI) epithelial cell line, T84, in the basolateral-to-apical direction, but not the reverse, unless epithelial tight junctions were disrupted. This observation suggested that NSCFs produced by *C. albicans* and other yeast species may influence host-pathogen interactions at the GI mucosa by inducing phagocytic cell infiltration.

The role of β_2 -integrins in phagocytosis and killing of *C. albicans* was confirmed using neutrophils isolated from calves with bovine leukocyte adhesion deficiency (BLAD). BLAD neutrophils displayed decreased phagocytosis and killing capabilities, supporting claims that β_2 -integrins are the primary PMN adhesins for *C. albicans*.

To examine the differences in adhesion of *Candida* species to human GI epithelium, radiolabeled yeast were allowed to adhere to T84 and Caco2 monolayers. The adhesion to T84 monolayers correlated with the reported prevalence of *Candida* spp. in the human GI tract and the adhesion to Caco2 monolayers was similar, although *C. glabrata* displayed greater adherence than other yeast tested. *C. tropicalis* adhered poorly to both cell lines in contrast to exfoliated buccal and vaginal epithelial cells, suggesting differences in expression of cell adhesion molecules on different epithelial cell types and/or *Candida* species. Therefore, human intestinal epithelial cell lines are a useful *in vitro* model system to investigate the initial steps of colonization of the human GI tract.

CHAPTER 1

INTRODUCTION

Candida albicans is recognized as a human opportunistic fungal pathogen that can cause a wide range of diseases ranging from superficial candidiasis of the skin or mucosa to life-threatening hematogenously-disseminated disease. *C. albicans* is commonly a member of the normal flora of the lower gastrointestinal (GI) tract of healthy individuals (72). It is also regularly found in the oral cavity (15), the female genital tract (32,372), and occasionally on the skin (297). The majority of *Candida* infections result from the uncontrolled growth of the individuals own endogenous yeast due to failure or suppression of the host's immune system (327,430). Therefore, the ability of *C. albicans* to adhere to and grow as a commensal on mucosal surfaces is considered a putative virulence trait that will be discussed in detail.

In the 1980's, there was a dramatic rise, close to 500%, in large U.S. teaching hospitals, of *Candida* species nosocomial blood stream infections (BSIs), (25). This rise directly correlated with the increased numbers of immunocompromised individuals due to advances in modern medicine. Currently, *Candida* spp. are the fourth leading cause of both nosocomial BSIs (315) and nosocomial urinary tract infections (UTIs) (175) hospital wide. *Candida* spp. are the number one cause of UTIs in intensive care unit patients (176). *C. albicans* is the most commonly isolated species for nosocomial BSIs and UTIs, although an increase in other *Candida* spp. has recently been documented (159,316,413). The outcome for patients with disseminated candidiasis is bleak. Diagnosis is difficult due to the low recovery rate of yeast from blood cultures (349,383) and there are only a few antifungal agents available for treatment (433). In non-neutropenic patients with systemic candidiasis, the crude mortality rate is reported to be 55-70% and the attributable mortality 26 to 49% (413,431). In neutropenic patients, the attributable

morality may be as high as 94% (148,243). These statistics emphasize the need for better antifungal agents, diagnostic tests, and understanding of the host's defenses against these opportunistic pathogens.

For *Candida* spp., the switch from a commensal to a pathogen is uncommon without suppression of the host's immune system, innate or cellular mediated (250,251,264,348,437). There is now a large population of people with compromised immune systems that are being kept alive longer due to current advances in modern medicine. Primarily, these are people with impaired host defense mechanisms due to either primary disease states (e.g., AIDS, cancer, or diabetes), the use of broad-spectrum antibiotics that disrupt the mucosal normal flora, immunosuppressive therapeutic regimens (e.g., cytotoxic chemotherapies and immunosuppression for organ transplantation) (430), and invasive devices and procedures (176). Therefore, it is not surprising that the incidence of opportunistic fungal infections has increased dramatically over the past two decades.

Polymorphonuclear leukocytes (PMNs) have been shown to be the primary component of the host's innate immune defenses against disseminated candidiasis in *in vitro* studies (104), animal models (129), and studies of neutropenic patients (250). A better understanding of the interactions between *C. albicans* and professional phagocytic cells would provide valuable insights into how the body protects itself against this opportunistic pathogen. My research dissertation has focused on the recognition of *C. albicans* by professional phagocytic cells. The primary focus was on small molecular weight non-serum dependent chemotactic factors (NSCFs) released from *C. albicans* blastoconidia that stimulated chemotaxis of both human PMNs and a mouse macrophage cell line, J774. An *in vitro* model of the intestinal epithelium (310,311) was used to investigate the ability of the NSCF to induce PMN transmigration across the intestinal epithelium (simulating the transmigration of PMNs into the GI lumen). I also extended

the use of this model to investigate the ability of *C. albicans* to colonize the human GI tract. Topics that will be discussed in this introduction include: the biology of *C. albicans*, host anti-*Candida* defense mechanisms, with an emphasis on PMNs, and the adhesion to and colonization of the host's mucosal surfaces, particularly the GI tract, by *C. albicans*.

Biology of *Candida albicans*

Candida spp. are classified in the Kingdom fungi, subdivision Deuteromycotina, also known as the Fungi Imperfecti. They are further classified in the class Blastomycetes, the order Cryptococcales, and the family Cryptococcaceae (217). *C. albicans* is a polymorphic fungus that can grow as blastoconidia, pseudohyphae and true hyphae *in vivo* and *in vitro* (297). All three morphological forms are found at the foci of infection (297). A fourth form, chlamydoconidia, is also commonly observed *in vitro* (388) and has been reported *in vivo* (77). The terms blastoconidia and yeast will be used synonymously throughout this thesis. In addition, the term hyphae will be used to describe filamentous forms of *Candida* unless the use of pseudohyphae or germ tubes were explicitly stated in the work cited. Many different environmental conditions have been found to influence hyphal formation, such as yeast concentration, temperature, pH, nutrient availability, carbohydrate type and concentration, and serum (147). The ability to switch from yeast to hyphal growth is considered to be a putative virulence trait due to the penetrative nature of the hyphae and environmental adaptability (147). The diameter of *C. albicans* blastoconidia and hyphae is in the range of 3 to 6 μm . *C. albicans* forms small, white to cream colored, smooth colonies on Sabouraud dextrose agar at 35 to 37°C. *C. albicans* also has the ability to form a variety of alternate colony morphology phenotypes, such as an opaque phenotype (374), and tends to spontaneously switch between the different phenotypes at a frequency of $\sim 10^{-7}$ (368). These alternate

phenotypes are hypothesized to enable *C. albicans* to adapt to different environmental conditions *in vitro* and *in vivo* (7). Isolates of *C. albicans* fall into two major serotypes, A and B (156). The serotypes are distinguished by the structure of the side chain oligosaccharides attached to the backbone α 1,6 mannosyl residues of the mannoprotein complex in the cell wall (290). *C. albicans* has a diploid genome consisting of 8 chromosomes and does not appear to go through meiosis. However, the identification of genes in *C. albicans* that are homologous to *Saccharomyces cerevisiae* genes involved in the mating pathway of haploid cells, suggests that *C. albicans* once had a sexual cycle (232).

Host Defense Mechanisms against *Candida albicans*;
Emphasis on Polymorphonuclear Leukocytes

In 1883, the Russian zoologist, Elie Metchnikoff, presented his phagocyte theory of host defense (34,393). The term phagocyte (phagos (to eat) and cyte (cell)), coined by Elie Metchnikoff and Karl Claus, was used to describe the mesodermal amoeboid cells of starfish larvae that acted in host defense against an intruder, a rose thorn that was placed under the larva's skin. Later, Metchnikoff's observations of mammalian leukocytes, polymorphonuclear leukocytes (PMNs) and macrophages, actively engulfing invading organisms, led to the formulation of his theory of immunity. He proposed that phagocytosis provided the main, if not the only, defense against infection.

We now know that host defense mechanisms are much more complex than envisioned by Metchnikoff; being composed of both innate and acquired components. The impairment of innate resistance is associated with hematogenously disseminated candidiasis, while acquired resistance abnormalities predisposes one to superficial candidiasis. Components of innate immunity against *Candida* are leukocytes, both PMNs (or neutrophils) and macrophages/monocytes, mucosa normal flora, complement

cascade, and fungistatic or fungicidal compounds contained in fluids, such as serum, saliva and respiratory secretions. Yet, this simplistic view of host resistance to systemic candidiasis is also naïve. In animal models, the cellular mediated immune (CMI) response has been found to influence the recovery of animals with systemic candidiasis (338). In addition, the humoral response to *Candida* was initially thought not to play a part in host defense. It is now known that antibodies directed against specific cell wall epitopes of *C. albicans* are protective against systemic and vaginal candidiasis in animal models (61,153,155).

The PMN functions as the primary effector cell against *C. albicans* invasion. In immunologically normal individuals, a inflammatory response consisting mostly of PMNs is the hallmark of the majority of *Candida* lesions. Together with monocytes and tissue macrophages, PMNs modulate and carry out many of the major functional responses of the innate immune system. For candidiasis, these include: recognition of and rapid mobilization to the foci of infection, modulation of the acquired host response, and ultimately killing of the invader. PMN anti-*Candida* functions are also influenced by the CMI system. In addition, *C. albicans* has developed mechanisms to evade these immune responses. Each of these processes will be considered separately. I will limit this discussion to PMN anti-*Candida* defense mechanisms. Monocytes and macrophages will be discussed only when necessary.

Mechanisms of *C. albicans* dissemination and adhesion to the vascular endothelium

Once *C. albicans* has reached the blood stream, adhesion to the vascular endothelium is critical for invasion of target organs. Of the *Candida* spp. tested, *C. albicans* displayed the greatest capacity for adhesion to the endothelium of porcine blood vessels (202) and cultured human umbilical vein endothelial cell (HUVEC) monolayers (342) *in vitro*. Both non-specific and specific mechanisms are thought to be involved in

adhesion to the endothelium. Hydrophobic interactions between *C. albicans* and host cells most likely acts as the predominant adhesive force during initial contact (158). *C. albicans* germ tubes display high cell surface hydrophobicity (CSH) (157) and the transformation from yeast to hyphal growth was shown to correlate with increased adhesion to host surfaces (341). Yet, evaluation of the contribution of *C. albicans* CSH to adhesion is problematic (158). This is due to the ability of *C. albicans* to quickly modulate its' CSH, and the difficulty to strictly evaluate the contribution of CSH to adherence separate from other adhesion mechanisms. In addition, hydrophobic yeast and hyphal cells tend to clump which may create artificially high adhesion values.

Multiple surface antigens on *C. albicans* have been reported to be involved in specific adhesion to endothelial cells. Adhesion of *C. albicans* to both human epithelial (30) and endothelial monolayers (151,443) was found to be mediated, in part, by the complement receptor 3-like receptor (CR3-like receptor) expressed by *C. albicans*. CR3-like receptor recognizes the complement components iC3b and C3d (102,105), ligands for mammalian CR3. Based on cross-reactivity with monoclonal antibodies (mAbs), the CR3-like receptor is thought to be antigenically similar to CD11b and CD11c, the α -subunits of the mammalian β_2 -integrins, CD11b/CD18 (CR3) and CD11c/CD18 (p150,95) (102,105,143,151,165). Mammalian β_2 -integrins are expressed in varying degrees by all human leukocytes and lymphocytes and are involved in cell-cell and cell-matrix adhesion functions (130). CR3-like receptor expression appears to be greatest on pseudohyphae and hyphae (102,162) with some activity on blastoconidia (143). The expression is up-regulated at 30°C (105,443) and when yeast are grown in high glucose concentrations (166). A variety of MW size mannoproteins from *C. albicans*, ranging from approx. 40- to 185-kDa, are recognized by anti-CR3 mAbs (103,105,166).

Another *C. albicans* surface mannoprotein recognizes the complement component C3d and has been given the name CR2-like receptor (231,352). Although, no antigenic similarities to mammalian CR2 (CD21) have been demonstrated. CR2 is a single transmembrane glycoprotein expressed on B cells and follicular dendritic cells (333). Like the CR3-like receptor, the CR2-like receptor has been postulated to be involved in endothelial cell adhesion (103,415). Surface expression of CR2-like receptor was reported on hyphae but not blastoconidia *in vivo* and *in vitro* (182). A C3d-binding 55- to 60-kDa glycoprotein was purified from *C. albicans* and was able to inhibit binding of C3d-coated erythrocytes to pseudohyphae (352). After deglycosylation, the protein had an apparent molecular weight of 45-kDa by polyacrylamide gel electrophoresis. Purified CR2-like receptor also inhibited rosetting of hyphae with erythrocytes coated with iC3b, raising the possibility that the CR2-like and CR3-like receptors are actually one protein with alternate glycosylation (415).

Definitive proof of an integrin-like protein expressed on *C. albicans* and its similarity to mammalian integrins has come from the recent cloning of the *C. albicans* gene $\alpha INT1$ (132,133). An open reading frame was predicted to encode an approximately 188-kDa polypeptide. The structure of $\alpha Int1p$ is similar to mammalian integrins with a single transmembrane domain, I domain, divalent cation-binding motifs, and an arginine-glycine-aspartic acid (RGD) sequence. $\alpha INT1$ was found to be involved in the yeast/hyphal transition and adhesion to epithelial HeLa cell monolayers (132,133). In addition, *C. albicans int1/int1* strains were less virulent in a systemic murine model (132). Expression of $\alpha INT1$ in haploid *S. cerevisiae* induced the production of germ tubes and adhesion to HeLa monolayers (132). The ligand for $\alpha Int1p$ is unknown.

Of interest is the predicted RGD sequence contained in the putative I domain of $\alpha Int1p$. Mammalian integrins mediate cell adhesion by binding to proteins that contain this triamino peptide sequence (343), such as extracellular matrix (ECM) proteins

(fibronectin, laminin, and collagens I and IV), C3 fragments and fibrinogen. The RGD flanking sequences, and possibly other binding sequences, appear to direct the specificity of the different integrins for various ligands. Yet, the exact role of the RGD-sequence in mammalian receptor-ligand recognition has been challenged. CR3 recognition of iC3b does not appear to take place through the RGD-sequence. Site directed mutagenesis of RGD to AAA did not effect binding of CR3 to iC3b (392). The RGD-containing ligands may actually bind to integrin associated protein (CD47) on PMNs and influence CR3 activity via this receptor (409). Nevertheless, it appears that some pathogens may adhere to host cells or the extracellular matrix by recognition of RGD and RGD-like sequences. Adhesion of *C. albicans* to epithelial cells (29) and ECM proteins (206,312) appears to be inhibited by RGD-containing peptides *in vitro*. In addition, pre-treatment of *C. albicans* yeast with RGD-containing peptides increased the hepatic fungicidal activity in an isolated perfused mouse liver model (351), and a synthetic peptide containing the RGD sequence protected rabbits against an IV challenge with *C. albicans* (207). These results indicated that receptors expressed on *C. albicans* that recognized RGD-containing ligands may play an important role in pathogenesis.

Mannoproteins expressed on *C. albicans* that have been implicated in adhesion to ECM proteins include a fibronectin-binding protein (204,206), and a laminin-binding protein (43). Anti-human β 1-integrin mAbs recognized a 60-kDa glycoprotein isolated from *C. albicans* that was implicated in adhesion to fibronectin, laminin and type I collagen, suggesting *C. albicans* expressed a β 1-integrin homologue (205). The collagenous domain, not the RGD-containing domain, of fibronectin has been suggested to mediate binding of fibronectin by *C. albicans* (292,360). However, peptides containing the RGD sequence inhibited binding of *C. albicans* to immobilized fibronectin but not to other ECM proteins (206,312).

The *C. albicans* cell surface glycoproteins, Als proteins (agglutinin like sequence), are homologues of the *S. cerevisiae* agglutinin protein- α 1 and have also been shown to be involved in endothelial and epithelial cell adhesion (167). *S. cerevisiae* expressing the *C. albicans* gene, *ALS1*, displayed increased adherence to HUVEC and FaDu, an oropharyngeal epithelial cell line, monolayers (128). Additionally, increased adherence to all ECM proteins tested was noted with *S. cerevisiae* expressing the *C. albicans* gene *ALA1*, a gene with significant amino acid sequence homology to *ALS1* (138).

The interaction of *C. albicans* with serum proteins may influence the binding of *C. albicans* to host cells and/or ECM proteins. Yeast grown in the presence of hemoglobin were found to display enhanced adhesion to immobilized ECM proteins, fibronectin, laminin, fibrinogen and type IV collagen (445). Fibrinogen was found to bind to *C. albicans* through a 58-kDa fibrinogen-binding mannoprotein (60). Activated platelets, that are found at sites of endothelial damage, bind fibrinogen through the β_3 -integrin, GPIIb/IIIa (1), and have been shown to bind to *C. albicans* germ tubes that are coated with fibrinogen (189). The adhesion of *C. albicans* to EDTA-treated HUVEC monolayers was enhanced when platelets were added to the system (203). The yeast were found to bind to platelet aggregates formed at sites where ECM proteins were exposed. Although not investigated, this yeast-platelet interaction was most likely mediated through the binding of fibrinogen by both the yeast and platelets.

C. albicans Invasion of Vascular Endothelium

Penetration of HUVEC monolayers by *C. albicans* was shown to occur by phagocytosis of yeast or germinated cells by the endothelial cells (342). Of *Candida* spp. tested, only *C. albicans* was engulfed by the endothelial cells, suggesting the phagocytosis was specific for *C. albicans*. Dead yeast and germinated cells were

phagocytosed by the endothelial cells, but only live, engulfed yeast caused significant cellular damage to the endothelial cells. Damage was found to occur by an iron-dependent, reactive oxygen intermediates-independent mechanism (117,124). The addition of PMNs (104) or pretreating HUVEC monolayers with IFN- γ (125,169) inhibited cellular damage by *C. albicans*. IFN- γ prevented phagocytosis of yeast by the endothelial cells and, therefore, cellular damage. In addition, phagocytosis of *C. albicans* stimulated endothelial cell responses that had immunomodulating activity. Filler and colleagues showed *C. albicans* induced the synthesis and release of prostaglandins (prostaglandin I₂ (PGI₂), PGF_{2 α} , and PGE₂) from HUVEC monolayers (116,117). PGI₂ was shown to inhibit adhesion of *C. albicans* yeast to endothelial monolayers and also to break up platelet aggregates to which *C. albicans* yeast were strongly adherent (201). Klotz showed that *C. albicans* may actually inhibit the release of PGI₂ from endothelial cells at early time points but acted as a stimulant for PGI₂ production only at later time points during the infection (201). *C. albicans*-infected HUVECs displayed upregulated synthesis and surface expression or release of many proinflammatory molecules: E-selectin, ICAM-1, vascular cell adhesion molecule 1 (VCAM-1; CD106), interleukin-6 (IL-6), IL-8, and monocyte chemoattractant protein 1 (118). Although not tested, the damaged endothelium probably also releases formylated peptides that induce leukocyte chemotaxis (59).

PMN Recruitment to the Foci of Infection

To get to the foci of infection, PMNs must adhere to the endothelium and then emigrate from the microvasculature into the tissue by amoeboid-like movements, a process termed transendothelial migration or diapedesis. PMN adhesion is dependent upon the activation status of the endothelium. Addition of *C. albicans* to endothelial monolayers results in activation of the endothelium (116,118). The initial PMN adhesive

event results from weak interactions between members of the selectin family. Activation of endothelial cells results in transient expression of P-selectin within minutes from internal stores (260). L-selectin (CD62L) (377) and P-selectin glycoprotein ligand-1 (CD162) (16,261) expressed on PMNs bind to carbohydrate ligands, sialyl Lewis X and sialyl Lewis A antigens, on sialoproteins E- and P-selectins expressed on the endothelial cells (CD62E and CD62P respectively). These interactions result in the tethering and rolling of PMNs on the endothelial surface (306). Stimulated endothelial cells rapidly synthesize and express platelet activating factor (PAF). PAF binds to the PAF receptor (PAFR) on PMNs. Both binding of P-selectin to its respective ligand and PAFR to PAF on the endothelium results in inside-out signalling of β_2 -integrins, CD11a/CD18 (lymphocyte function-associated molecule-1), CD11b/CD18 and CD11c/CD18 (234,235), expressed on the PMN surface. This results in a conformational change of the β_2 -integrins allowing binding to their respective ligands, ICAM-1 and ICAM-2 (CD102), and results in firm adhesion of the PMN to the endothelium. After PMN activation, L-selectin expression is down-regulated and shed into the circulation. Stimulation of endothelium *in vitro* by LPS, TNF- α , IL-1 β , IL-4, and IFN- γ results in up-regulation at the transcriptional level of ICAM-1, VCAM-1, P-selectin, and E-selectin (306). P- and E-selectin expression reaches a peak at 3 – 5 hours after endothelial activation. While VCAM-1, involved in lymphocyte and monocyte transendothelial migration, expression peaks at approximately 6 hours and ICAM-1 at about 12 hours after stimulation. Although, the intensity of ICAM-1 up-regulation varies between different vascular beds *in vivo* (306). Activation of PMNs by IL-8, formylated peptides, and leukotriene B₄ also results in β_2 -integrin activation. Transendothelial migration of PMNs up the chemotactic gradient toward the foci of infection is dependent upon CD11a/CD18 and CD11b/CD18 adhesive events; selectins do not seem to be necessary (306).

The cell wall of *C. albicans* is an excellent activator of the complement cascade (210). Activation of the complement cascade results in the production of the powerful inflammatory mediators C3a, C5a, and the less potent C4a. These mediators increase vasopermeability for inflammatory cell infiltration. C3a and C5a display strong chemotactic activity for PMNs, monocytes and macrophages. In addition, numerous other biological effects on PMNs have been attributed to these proteins (227), such as induction of the oxidative burst, release of lysosomal enzymes, β_2 -integrin surface expression, and release of inflammatory cytokines IL-1, IL-6, IL-8 and TNF- α . The release of compounds from *C. albicans* chemotactic for phagocytic cells may also play a role in directing cellular infiltration. A number of studies have shown that *C. albicans* produces non-serum dependent chemotactic factors (49,50,84,401,405), and my research has shown *C. albicans* produces chemotactic factors that can stimulate chemotaxis of both human PMNs and the mouse macrophage cell line, J774.

Leukocyte Adherence to *C. albicans*

PMNs take up *C. albicans* yeast into phagosomes that fuse with both granules and lysosomes to form the phagolysosome in which the PMN attempts to kill and digest the yeast (248). Filamentous forms of *C. albicans* are too large to be fully engulfed by phagocytic cells. Instead, the phagocytic cells attach to and spread over the cell surface (91), and degranulation occurs at the site of PMN contact with the hyphal cell surface. This is termed "frustrated phagocytosis". PMN adherence to *C. albicans* is dependent upon the recognition of both fungal- and host-derived components. Neither normal nor frustrated phagocytosis requires *C. albicans*' cell wall opsonization (91,313), but optimal adhesion, and therefore phagocytosis, occurs with opsonization by factors contained in fresh human serum (226). The cell wall of *C. albicans* is a potent stimulator of the alternative complement cascade, and anti-*Candida* antibodies and the mannose binding

protein (MBP) found in human serum can activate the classical complement cascade. This results in the deposition of C3b, iC3b and C3d on the cell surface (211). PMNs express receptors for C3b and iC3b, CR1 (CD35) and CR3 (CD11b/CD18), respectively. Large internal pools of CR1 relocate to the cell surface upon PMN activation (113). In addition, upon activation of PMNs, CR3 undergoes a conformational change (inside-out signalling discussed above) resulting in enhanced ligand binding affinity. Although, binding of polysaccharide ligands to CR3 does not seem to require inside-out signaling. The receptors for the Fc region of IgG, Fc γ RIIa (CD32), Fc γ RIIIb (CD16), and IgA, Fc α RI (CD89), are normally expressed by human PMNs and can be up-regulated with inflammatory factors, such as IFN- γ (273). Human PMNs do not usually express Fc γ RI (CD64), the high-affinity receptor for IgG, but expression is upregulated with IFN- γ and granulocyte-macrophage-colony stimulating factor (GM-CSF) (314,339). Cooperation between Fc γ Rs and CR3 results in enhanced adhesive capabilities of PMNs (215). Additionally, chemoattractant factors, such as formylated peptides, C5a, LTB₄ and PAF, stimulate increased surface expression of β_2 -integrins, resulting in enhanced adhesion of PMNs to the vascular endothelium (402), and presumably, a greater numbers of PMNs at the foci of infection.

A variety of non-opsonic recognition systems are used by phagocytic cells to recognize *C. albicans* consisting of lectin-carbohydrate, protein-protein, and hydrophobin-protein interactions (300). In addition to iC3b, other ligands for CR3 include β -glucans (395), ICAM-1, factor X (89) and fibrinogen (395). As previously stated, *C. albicans* can bind to ECM proteins found in serum and the basement membrane and exposure to hemoglobin enhances the binding to several of these ECM proteins (445). Host effector cells may recognize ECM proteins bound to the cell wall of *C. albicans* by integrins, such as CR3 (378).

Unlike PMNs, macrophages and monocytes express surface receptors that are specialized for nonopsonin-mediated phagocytosis of targeted particles by recognition of terminal carbohydrate moieties (380). These receptors have been found to be important for adherence to *C. albicans* (111,247). Macrophages, but not monocytes (363), express the macrophage mannose receptor (MMR) that binds to terminal mannose, fucose, *N*-acetylglucosamine, and glucose residues (273). In addition, monocytes express a β -glucan receptor implicated in adhesion and phagocytosis of *C. albicans* (174).

Serum mannose binding protein (MBP), C-type lectin secreted by the liver, is thought to play an important role in the initial stages of host defense against *C. albicans* (390). MBP recognizes *C. albicans* and activates the classical complement cascade in a similar manner as C1q (177,390). In addition, MBP enhances phagocytosis by binding to both mannose residues on the microorganism and to the C1q receptor on both macrophages and PMNs (300,394).

Phagocytic Cell Killing Mechanisms:

The antimicrobial killing arsenal of PMNs contains both oxidative and non-oxidative mechanisms that work in concert. The proteins that are involved in both mechanisms are contained within the PMN granules. There are 3 types of granules in PMNs (70): primary (azurophilic), secondary (specific), and tertiary. After ingestion of the microorganism into the phagosome, the granules and lysosomes fuse with the phagosome and release their deadly contents (24,329). In "frustrated phagocytosis", the fusion of granules takes place with the plasma membrane in direct contact with the fungal cell surface. Granule constituents are released into the space directly between the plasma membrane and the hyphal cell. Some secondary and tertiary granule products that may contribute to killing or collateral injury, such as lactoferrin (LF) (41,237) and myeloperoxidase (MPO) (47,324), are also released into the extracellular milieu.

Phagocytosis of invading microorganisms by PMNs and macrophages occurs concurrently with a large increase in the consumption of oxygen, termed the "respiratory burst" (21). This is due to the production of massive amounts of superoxide ($O_2^{\bullet-}$) by the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase) complex found in phagocytic cells (330). Monocytes and macrophages display a greater respiratory burst than PMNs, yet they convert less of the oxygen into superoxide than PMNs (86). Reduction of molecular oxygen occurs by the following reaction: $NADPH + 2O_2 \rightarrow 2O_2^{\bullet-} + NADP^+ + 2H^+$. The active NADPH-oxidase complex is composed of several different components (87). The complex is dissociated in unstimulated cells. The components are found in the plasma membrane (flavocytochrome b558 and Rap1A), and cytosol (p47-*phox*, p67-*phox*, p40-*phox*, Rac1/2) compartments of the cell (87). Flavocytochrome b558 is a heterodimer composed of a large and small subunit, gp91-*phox* and gp22-*phox* respectively, that associate with a flavin-adenine dinucleotide molecule. In non-activated PMNs, the majority of flavocytochrome b558 is found in the secondary granules membrane (90%), with a small amount in the plasma membrane (10%) (179). When activated, the components of the NADPH-oxidase translocate to the plasmalemma that forms the phagosome and form a large multicomponent activated complex that is able to reduce molecular oxygen to superoxide (42,87,179).

Due to the inherent instability of $O_2^{\bullet-}$, it rapidly dismutates to form hydrogen peroxide (H_2O_2). In the presence of a halide ion, such as Cl^- , MPO converts H_2O_2 to OCl^- which can react with amines to form long-lived *N*-chloramines that have potent antimicrobial activity. OCl^- can further interact with $O_2^{\bullet-}$ to form extremely reactive hydroxyl radicals (OH^{\bullet}) (340). Studies suggest that MPO released into the extracellular milieu from PMNs enhances macrophage anti-*Candida* activity (223). In addition, inducible nitric oxide synthetase has been shown to be produced by PMNs in primary granules (110). Through MPO-dependent pathways, activated human PMNs were shown

to convert NO^- into the inflammatory oxidants NO_2Cl and NO_2^\bullet *in vitro* (106).

The importance of PMN oxidative killing mechanisms is most apparent in individuals with chronic granulomatous disease (CGD) (274). CGD patients have a defect in one of the components of the NADPH-oxidase and are unable to make superoxide. They suffer from chronic and recurrent bacterial and fungal infections. *In vitro*, CGD PMNs display decreased killing efficiency of many microorganisms, however the majority of recurrent infections are caused by those capable of producing catalase (245). Approximately 20% of CGD patients experience fungal infections, primarily by *Aspergillus* spp., that includes pneumonia and/or widely disseminated disease with bone involvement (71). Candidiasis appears to be relatively rare in CGD patients.

There are two mouse models of CGD, gp91- (319) and p47-null (172) mice (gp91^{phox-/-} and p47^{phox-/-} mice, respectively). Both display similar responses to challenge with pathogens that normally cause recurrent infections in CGD patients (245,269). Although, p47^{phox-/-} mice, unlike gp91^{phox-/-} mice, develop spontaneous infections (172). In gp91^{phox-/-} mice, enhanced proinflammatory cytokine production in response to intranasal challenge with *A. fumigatus* and chronic bronchopneumonia with granuloma formation after administration of sterile hyphae into the lungs was observed, suggesting that the pathology associated with CGD was also due to dysregulation of the inflammatory response (269). In addition, catalase deficient *Aspergillus nidulans* was shown to be as virulent as wild type in p47^{phox-/-} mice, suggesting that the role of catalase as a fungal virulence factor in CGD patients should be reevaluated (64).

In addition to CGD PMNs, PMNs derived from individuals with complete MPO-deficiency demonstrate impaired killing of catalase-positive organisms and *Candida albicans in vitro* (22). MPO-deficient individuals are usually asymptomatic (22). However, there are reported cases of recurrent *Candida* infections in MPO-deficient patients, but infections are usually associated with additional immunocompromising

conditions, such as diabetes mellitus (218,293). Enhanced production of superoxide anion (140), alternative metabolism of H_2O_2 by other systems within the phagolysosome, such as the Haber-Weiss reactions (140), the production of reactive nitrogen species by iNOs (278), and normal eosinophil peroxidase activity (140) are thought to compensate for the loss of MPO activity.

Recently, a MPO-deficient mouse was generated (14). These mice displayed enhanced susceptibility to pneumonia and death following intratracheal infection with *C. albicans*, and increased rate of dissemination of intraperitoneally injected *C. albicans*. The authors suggested that MPO-deficient mice were unable to compensate with other oxygen-dependent microbicidal systems *in vivo*. However, the relatively rare occurrence of candidiasis in MPO-deficient patients, without an additional immunocompromising condition, suggest that other microbicidal systems can compensate for the loss of MPO activity.

PMN candidacidal responses are thought to consist of both non-oxidative and oxidative mechanisms, and may explain why *C. albicans* infections are rarely observed in MPO-deficient and CGD patients. Non-oxidative mechanisms were found to severely damage hyphal cell wall, although the addition of oxidative mechanisms to the *in vitro* system resulted in enhanced anti-fungal activity (66). *In vitro*, *C. albicans* yeast appeared to be susceptible to non-oxidative killing by PMN granule extracts at pH 5.5 to ≤ 7.5 (299), values thought to be necessary for the action of some non-oxidative killing mechanisms (255,358), whereas *A. fumigatus* conidia were highly resistant throughout the pH range tested (pH 5.5 to 8.5). In addition, CGD PMNs were found to inflict a significant amount of damage to *C. albicans* hyphal cell walls *in vitro* (66). Although, the addition of oxidative-killing mechanisms to CGD PMNs significantly enhanced damage to the cell wall, as well as DNA, of the hyphal cells (66)

LF is an iron binding protein that plays a role in both non-oxidative and oxidative killing mechanisms. Sequestering of Fe^{3+} inhibits the reduction of Fe^{3+} to Fe^{2+} by $\text{O}_2^{\bullet-}$ that results in the reduction of H_2O_2 to OH^\bullet by the metal ion catalyzed Haber-Weiss reaction ($\text{O}_2^{\bullet-} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^\bullet + \text{OH}^- + {}^1\text{O}_2$) (70), while iron saturation of LF results in enhancing PMN superoxide production in response to *f*MLF (131). Prevention of OH^\bullet formation by LF is believed to allow a certain level antimicrobial activity by PMNs while minimizing the harm to the surrounding tissue.

In vitro, PMNs can inhibit growth of *C. albicans* without direct contact (373). Release of LF and other cytoplasmic and granular proteins from intact or lysed PMNs have been suggested to account for this observation. The anti-*Candida* effects of LF are well documented (28,301,304,305,376). LF is also produced by mucosal epithelial cells and is found in external secretions, such as saliva, where it may play a pivotal role in early host defense (53). Competition for iron is thought to be one way LF exerts this inhibitory effect; only 20% of LF in saliva has iron bound to it. Apolactoferrin (iron free lactoferrin) exerted fungicidal activity *in vitro* (376). Lactoferricin B, a peptide derived from the *N*-terminal region of LF, bound to the cell surface of *C. albicans* and stimulated what appeared to be an autolytic response (28). LPS activated PMNs displayed enhanced anti-*Candida* activity that was correlated to the secretion of LF (304). Furthermore, the addition of LF to PMNs, *in vitro*, increased the inhibitory growth effect of human PMNs on *C. albicans* (301). *C. albicans* cell wall mannoproteins were found to be strong stimulators of LF release from human PMNs *in vitro* (305). LF has also been shown to exert immunomodulatory effects on PMNs. PMNs exposed to human lactoferrin, *in vitro*, exhibited increased adhesion (303), chemokinesis and *f*MLF stimulated superoxide production (131). LF released from PMNs may also affect mononuclear leukocytes. LF treated human monocytes and monocyte-derived macrophages did not show increased

motility or *f*MLF stimulated superoxide production (131), yet LF was found to directly stimulate phagocytic and cytotoxic properties of macrophages against microorganisms, such as *Trypanosoma cruzi* (230).

Other PMN granule and cytosolic compounds that were demonstrated have anti-fungal activity either alone or in combination with oxidative mechanisms include azurocidin (57), calprotectin (48,279), cathepsin G, elastase, defensins, and even lysozyme has been found to have weak fungicidal activity *in vitro* (90). Defensins are small polycationic peptides, 29-35 amino acid residues, that make up to 50% of the total protein content of the primary granules, 5-7% of total human PMN protein (40). They are not found in human monocytes or macrophages. Defensins have a broad spectrum of activity and have been found to have anti-*Candida* activity under suitable conditions *in vitro* and *in vivo* (225,434). It is thought that they insert themselves directly into the cell wall or plasma membrane of the microbe (432), although the exact mechanism of how defensins work is unknown.

T Helper Cell Response to *C. albicans* and Induction of PMN Anti-*Candida* Activity

Using inbred mouse strains, it was determined that the outcome of systemic or mucosal candidiasis depended upon the type of T helper (Th) cell response that developed (336,338). Protective host immune responses to *C. albicans* correlated with the development of a Th1 responses, whereas Th2 responses were associated with susceptibility. Th1 and Th2 type responses are differentiated by Th cell cytokine profiles that modulate the development and activity of immune effector cells. A Th1 cell-type cytokine pattern (IL-2, IL-12, interferon [IFN]- γ , and tumor necrosis factor [TNF]- β) skews the immune response toward CMI effector functions, such as activated cytotoxic cells. Alternatively, a Th2 cell-type cytokine pattern (IL-4, IL-5, IL-6, and IL-10) skews the immune response toward humoral mediated immunity effector functions. Although,

the Th cell cytokine patterns are much more complex than this, production of cytokines by Th cells that enhance the candidacidal activity of PMNs and monocytes/macrophages have been associated with protective host responses. Some cytokines originally thought to be associated strictly with a Th1 or Th2 response may actually be necessary for the development of both types of Th cells (288). For example, IL-4-deficient mice were unable to control systemic and GI *C. albicans* infections during late stages of disease, and IL-4 primed PMNs were necessary for IL-12 production (244). Also, some cytokines do not fit neatly into a particular category, such as TNF- α , GM-CSF, and IL-3. TNF- α and GM-CSF tend to mostly be associated with a Th1-type response, but may be present in either type (333).

In vitro, the treatment of phagocytic cells (PMNs and monocytes or macrophages) with Th1- or Th2-type cytokines has led to a greater understanding of cytokine-induced effects on anti-*Candida* functions by these cells. *In vitro*, PMNs readily engulf large numbers of *C. albicans* yeast, although the percent killed is relatively low (313). The killing efficiency is greatly enhanced when PMNs are activated by pro-inflammatory cytokines (18,96,335). In addition, PMNs not only respond to certain stimuli by releasing or upregulating the surface expression of preformed macromolecules, but are also able to synthesize many important proteins, including cytokines. This realization has changed the view of the PMN in early host defense from strictly an end effector cell to one with immunoregulatory functions as well. For simplicity, I will restrict my discussion of cytokine effects on anti-*Candida* PMN responses to human PMNs and only refer to information gained from murine models when necessary.

Supernatants from natural killer (NK) cells exposed to *C. albicans* blastoconidia containing the proinflammatory cytokines, TNF- α , IFN- γ and GM-CSF, simulated PMN anti-*Candida* activity as determined by growth inhibition (36,94,95). The effects of these cytokines on PMN anti-*Candida* functions were further characterized. Priming PMNs

with TNF- α *in vitro* resulted in increased expression of adhesion molecules (346), respiratory burst (114,196), degranulation (114), and enhanced chemoattractant receptor responsiveness due to an increase in heterotrimeric G-protein expression (259,354). TNF- α also acted as an autocrine factor by stimulating more TNF- α production from PMNs (95). IL-2 was found to stimulate the production of TNF- α (424), IL-8 (425), and LF (98) from PMNs. IFN- γ and TNF- α were synergistic in stimulating PMN anti-*Candida* activity *in vitro*, measured by fungal growth inhibition (97). In addition, co-incubating PMNs with *C. albicans* and TNF- α resulted in the production of the chemokines IL-8 and macrophage inflammatory protein 1- α (152). Yet, co-incubating PMNs with *S. cerevisiae* or zymosan and TNF- α resulted in IL-8 release only. Both IFN- γ and GM-CSF stimulated synthesis and expression of Fc γ RIa on PMNs (314,362,426), suggesting a role for PMNs in host defense at mucosal sites as well. Fc γ RIa expression was originally thought to be restricted to monocytes and macrophages. IFN- γ enhancement of PMN anti-fungal activity toward filamentous forms of *C. albicans* was found to be greater than both granulocyte-CSF (G-CSF) and GM-CSF (139).

Colony stimulating factors, G-CSF and GM-CSF, are potent stimulators of proliferation and differentiation of hematopoietic progenitor cells, and significantly increase the functional life span of PMNs. G-CSF and GM-CSF also amplify several important effector functions of mature PMNs that resulted in enhanced anti-fungal activity *in vitro*, including, superoxide production in response to chemoattractants (289,387,427) and *C. albicans* (331), Fc γ Rs and Fc α RIa synthesis and expression (195,328,426), CR3 expression (233,447), and synthesis of lipid mediators (323). *In vitro*, treatment of human PMNs with GM-CSF (444), but not G-CSF (332), resulted in enhanced growth inhibition or killing of *C. albicans*. Yet, PMNs isolated from healthy volunteers given G-CSF daily, for up to 5 days, did not display enhanced killing of *C. albicans*. Although, treatment did appear to prime the PMNs for sustained superoxide

production in response to *Candida* extracts (228) and modulate Fc γ RI and Fc γ RIIIb expression (107,265).

The morphological form of *C. albicans* used to stimulate murine peritoneal cavity PMNs, *in vitro*, was found to induce the selective release of IL-10 or IL-12 (334). *C. albicans* capable of undergoing the yeast to hyphal transition stimulated IL-10 production, whereas a non-germinating strain stimulated release of IL-12. Additionally, IL-12 production *in vivo*, that is thought to be from PMNs, correlated with enhanced resistance to candidiasis, while IL-10 with disease progression (335,337). Human PMNs stimulated with a mannose protein fraction (MP-F2) from the cell wall of *C. albicans* released IL-8, TNF- α , IL-6, and IL-1 β (62). But, if PMNs were stimulated with MP-F2 together with IFN- γ , IL-12 was also released. Therefore, the selection of the Th cell anti-*Candida* response appeared to be dependent upon the ability of PMNs to selectively produce IL-12 or IL-10, that in turn effects the immunomodulatory functions of PMNs.

Immunomodulation by *C. albicans*

The ability of a microorganism to elude the host's immune response ensures its ability to thrive and cause disease. In the case of *C. albicans*, it has evolved as a commensal of the mucosal surfaces in humans. Therefore, it has developed mechanisms that either inhibit or modulate the host's immune responses discussed above. The end result is colonization without disease, unless the host becomes immunocompromised and tips the balance in the favor of *Candida*.

As already discussed, *C. albicans* exhibits examples of molecular mimicry of mammalian proteins. The CR3- and CR2-like receptors appear to display anti-opsonic and anti-phagocytic activity by competing for ligand with the mammalian receptors (143,166). Enhanced phagocytosis by PMNs was observed when *C. albicans* was pretreated with the IgM mAb, Mo1, that recognizes the *C. albicans* iC3b receptor, and

decreased phagocytosis was observed when growth conditions were used that enhanced expression of CR3- and CR2-like receptors (143). Enhanced phagocytosis may actually have been the result of increased complement activation stimulated by Mo1, or recognition of the IgM by C1q receptors expressed on PMNs (394). Additionally, decreased phagocytosis of yeast grown under CR2- and CR3-like receptor expression enhancing conditions may have been due to alterations of expression of other surface antigens by *C. albicans*.

C. albicans is able to switch, at a high frequency and reversibly, between a number of different phenotypes that are distinguished by colony morphology, and in some cases by cellular morphology

(11,12,326). It has been hypothesized that the ability to switch between different phenotypes allows *C. albicans* to adapt to various environments, interact differently with host cells (190,375,412), and possibly display alternate virulence traits (190,191,411). It was shown in the white-opaque switching system, that switching was accompanied by the differential expression of phase-specific genes (270-272). In addition, opaque and white cells displayed differences in susceptibility to antifungal agents (141), and killing by PMN oxidative mechanisms (208).

A low molecular weight compound released into supernatants from live and dead *C. albicans* hyphae and pseudohyphae (crude hyphal inhibitory product or CHIP) inhibited contact between PMNs and fungal cells (92), and PMN superoxide production and degranulation in response to *f*MLF (370,371). Also, a high-molecular weight component in the same supernatants inhibited *Candida* killing by human PMNs *in vitro* (369). The low molecular weight compound was determined to be the immunomodulatory agent adenosine (369). Human PMNs express both the high affinity adenosine receptor, A1, and the low affinity receptor, A2 (82). Adenosine acting through the A2 receptor has anti-inflammatory effects on PMN function, such as decreased

superoxide production and adhesion to endothelium, and inhibition of phagocytosis. Yet, by acting through the A1 receptor, adenosine promotes PMN chemotaxis (81). In addition, these receptors influence phagocytosis mediated through Fc γ Rs in opposing ways (345). At low concentrations, adenosine acts through the A1 receptor to enhance PMN Fc γ R functions, but at high concentrations adenosine exerts an inhibitory effect on Fc γ R functions through the A2 receptor. The effect of adenosine released from *C. albicans* on Fc γ R mediated phagocytosis is unknown.

In addition to adenosine, mannoproteins released from the cell wall of *C. albicans* have been shown to affect leukocyte functions (187). A decreased or absent delayed-type hypersensitivity (DTH) response when stimulated with *Candida* antigens is often observed in patients with CMCC, disseminated candidiasis and recurrent vaginal candidiasis (381). This immunosuppression may be due in part to mannoproteins released from *C. albicans* cell wall. Within 15 minutes of interacting with human PMNs *in vitro*, *C. albicans* yeast released high molecular weight antigens, suggested to be mannoproteins (17). Treatment of mice with a mannan extract from *C. albicans* cell wall suppressed the DTH response to *C. albicans* cell wall mannan or glucan (99). Defective processing of mannan by CMCC patient's monocytes has been suggested to result in suppressed CMI responses in these patients (122). Mannan isolated from the serum of CMCC patients selectively inhibited PMN respiratory burst and MPO release (442). Zhang and Petty proposed that superoxide production was inhibited by mannan binding to CR3 on PMNs, thereby blocking transmembrane signalling (449). It has recently been shown that CR3 associates with and transduces cellular signals in a cis-acting fashion with a number of glycosylphosphatidylinositol (GPI)-linked receptors expressed on immune cells, such as Fc γ receptor IIIb (399). This interaction can be blocked with the carbohydrates D-mannose, α -methylmannoside and N-acetyl-D-glucosamine, resulting in the inhibition of important antimicrobial responses, such as superoxide production. A *C.*

albicans cell wall glycoprotein extract was found to inhibit CR3-mediated phagocytosis of *C. albicans*-yeast cells by macrophages (389). There is no experimental evidence that *C. albicans* cell wall mannanoproteins block the interaction of CR3 with other receptors on the surface of leukocytes or lymphocytes, although I believe this possibility merits investigation.

Candida albicans Colonization and Invasion of the Gastrointestinal Tract

The gastrointestinal (GI) tract is recognized as a portal of entry for hematogenous spread of *C. albicans* (73,188). *C. albicans* is commonly found as a commensal in the GI tract of humans and other animals (72). In healthy individuals, the carriage rate has been reported to be up to 80% (72,145,297). The ability of *C. albicans* to adhere to and colonize the mucosal surface of the human GI tract is considered a putative virulence trait (85). GI tract bacterial normal flora, alterations of the lumen environment by bacterial and host products, peristalsis, mucus secretions, and the continual renewal of enterocytes have all been suggested to prevent *C. albicans* colonization. An increased GI carriage rate of *C. albicans* has been associated with disturbance of the host's innate immunity, such as neutropenia, and the bacterial normal flora found in the GI tract (318,347,348,349). Although little is known concerning the maintenance of *C. albicans* as a commensal in the GI tract of healthy individuals (76,188,297,298), it is clear that dissemination from the GI tract is uncommon without immunosuppression of the host or perturbation of the normal flora (39,58,348,364). Therefore, a better understanding of both the mechanisms used by *C. albicans* for mucosal colonization and dissemination, and anti-*Candida* host defense mechanisms at the GI tract are warranted.

Mucocutaneous candidiasis is generally associated with *Candida* overgrowth on the upper GI mucosa (oral, pharynx and esophagus) (100,404), although immunocompromised individuals are also at risk of lower GI tract (duodenum, small and large intestine) involvement as well. Candidiasis restricted to the mucosa/submucosa is most often seen in patients with defects in CMI, such as AIDS (264). Disseminated candidiasis is sometimes seen in late stage AIDS patients (219), yet this is thought to be due to a decrease in phagocytic cell function as a result of generalized immunosuppression (75). Overgrowth of *Candida* spp. in the lower intestine and subsequent hematogenous dissemination to internal organs is most commonly associated with defects in innate immunity, particularly neutropenia (250,251).

C. albicans dissemination from the GI tract must require at least two steps: [1] the ability of *C. albicans* to persistently adhere to the mucosal lining, and [2] penetration of the lining either by an active or inactive process. Presumably, both of these steps are complex, i.e. composed of many individual components. The small intestine is the primary site of bacterial translocation out of the intestinal lumen (428), and in infant mice, *C. albicans* was shown to cross the epithelium in the upper thirds of the small intestine (115).

The cellular morphology of the intestine is complex (112,320). The small intestine is composed of crypt-villus units. The villi are finger-like projections surrounded by several crypts at their base. Multiple epithelial cell lineages (absorptive enterocytes or columnar epithelial cells, enteroendocrine cells, goblet cells and paneth cells) arise from multipotent stem cells contained within the crypts. As they mature, absorptive, goblet and enteroendocrine enterocytes migrate from the crypt to the tip of the villus. The enterocytes are polarized cells with an apical and basolateral surface with different morphology and surface antigen expression. The enterocyte apical surface is composed of microvillia (also called the brush border), 1,500 – 3,000 minute finger-like

projections per cell that are covered by the glycocalyx, a mucus glycoprotein layer. The intestinal lamina propria contains many diffusely scattered microscopic lymphoid nodules as well as macroscopic discrete domes of lymphoid nodules called Peyer's patches. The Peyer's patches are found throughout the small intestine with the greatest concentration in the ileum.

A high concentration of *C. albicans* is thought to be necessary for hematogenous dissemination from the GI tract (212). The GI tract bacterial normal flora is most likely the greatest barrier to *Candida* overgrowth at the mucosal surface (192,229,438). The use of broad spectrum antibiotics was shown to correlate with increased GI tract colonization by *C. albicans* in both humans (348,357) and animal models (192,193,229,438) and an increased susceptibility to hematogenous dissemination (430). The intestinal bacterial normal flora of a mature animal is estimated to contain up to 500 different species with anaerobes being the largest group (31). Dissemination from the GI tract was greatest in animals treated with antibiotics with good anaerobic activity and/or high GI concentrations (192,193). Therefore, the removal of a large number of normal flora members by broad-spectrum antibiotics may be detrimental to an immunocompromised host by allowing the overgrowth of opportunistic microorganisms, such as *C. albicans*.

The infant mouse was shown to be an informative animal model for investigating the mechanisms used by *C. albicans* for persistent colonization of the GI mucosal surface (76,161). Adult mice (conventional, pathogen- or germ-free, and gnotobiotic) given intragastric (i.g.) inoculation without antibiotic or immunosuppressive treatments are highly resistant to colonization by *C. albicans*, and eliminate the yeast from the GI tract within a relatively short period of time (69,229,406). This reflects that *C. albicans* is not part of the GI tract normal flora in the mouse. For unknown reasons, mice are resistant to *C. albicans* colonization. In the adult animals; persistent GI colonization was only

achieved with treatment, either singly or in combination, with irradiation (417), broad-spectrum antibiotics (193), corticosteroids (280), or cytotoxic drugs (438). High dissemination rates were also observed in these animals. Dissemination was most likely the result of damage to the integrity of the GI mucosa and induced granulocytopenia by these therapeutic agents (46,68,382). The infant mouse model offers the advantage of studying colonization *in vivo* with natural host barriers in place. If the infant mouse survived the initial inoculation, persistent colonization (at least 6 weeks post inoculum) could be achieved without other insults. Yet, it would be naive to assume all the natural host barriers to colonization are in place in the infant mouse. Because of their age, these mice are not immunocompetent, nor do they have a well established intestinal normal flora (322). Of course, humans are colonized early in life, probably as early as passage through the birth canal. Even so, it must be kept in mind that this animal model may not completely reflect the mechanisms involved in establishing long term colonization in humans.

Both specific and non-specific mechanisms are thought to be involved in *C. albicans* adhesion to the GI mucosa. Electron microscopy studies of infant mice inoculated i.g. with *C. albicans* showed yeast adhered to the mucosa by a variety of mechanisms: adhesion directly to the epithelium, adhesion to normal flora bacteria, co-adhesion to other yeast, and adhesion to or entrapment within the glycocalyx (76,193). Two strains of *C. albicans* that differed in persistence of colonization and systemic spread from the GI tract displayed similar distribution, determined by CFUs per gram of tissue, in the GI tract for the first 3 weeks after inoculation (115,321). Colonization of the upper and middle regions of the small intestine by these two strains was consistently lower than the stomach, ileum, cecum and colon (321). Yeast were commonly observed initially embedded within the glycocalyx of the intestinal epithelial surface, followed by what appeared to be an intimate association with the small intestine enterocytes

(115,321). An association of yeast with a single villus without attachment to adjacent villi was regularly observed (78). Abundant yeast cells were also observed associated with the cardiac-atrium fold of the stomach (74,76), the keratinized stratified squamous epithelium found between the stratified and glandular regions of the gastric mucosa (183), once again, suggesting specific fungal-host cell interactions.

Enterocytes are highly polarized with respect to morphology and surface antigen expression. Differences in yeast adhesion to the apical surface of intact murine GI epithelium explants were noted depending on the anatomical site the explants were derived from (263). These differences were lost when adhesion assays were done with cell suspensions made by trypsinization of explants. Although, surface adhesion molecules may have been altered by the trypsin influencing the adherence characteristics observed.

A number of *C. albicans* antigens have been implicated in adhesion to buccal and vaginal epithelium and extracellular matrix proteins, including: the fucose-binding adhesin (80,403), Int1p (132,133) Als proteins (128,138), laminin and fibronectin binding proteins (43,55), fibrinogen binding-proteins (60), and secreted aspartic proteinases (421). The role of these *C. albicans* adhesins in adherence to GI epithelium has not been investigated.

The exact mechanism of *C. albicans* passage through the mucosal surface in compromised patients is still unknown. Translocation of invading organisms through the intestinal epithelium may occur by two separate routes, transcellular or paracellular (238,428). One of the first studies to implicate the GI tract as a reservoir for disseminated candidiasis suggested passage through the GI lining by an intracellular pathway (212). Oral administration of a large dose of *C. albicans* blastoconidia (approximately 10^{12} yeast), to a healthy volunteer resulted in hematogenous dissemination from the GI tract. Dissemination occurred rapidly, approximately 2 hours

after ingestion yeast were found in the blood. Because of the short period of time from ingestion to dissemination, it was unlikely that translocation of the yeast occurred due to penetrative growth through the epithelium. The investigators postulated that movement of the yeast through the GI mucosa occurred by "persorption." The term persorption describes a process in which relatively large objects, up to ~10 μm , are transported through the intestinal lining by the host's own cells (414).

The infant mouse model supports both pathways for penetration of the GI lining. The rapid systemic spread that followed ig inoculation supports persorption as a mechanism of yeast translocation through the GI lining (115). Yeast could be found systemically by 30 min post inoculation (115). In addition, penetration of the microvillus barrier and intracellular invasion of the columnar epithelial cells in the jejunum by yeast cells was observed by electron microscopy 1 hour after inoculation (78). Thin fibrils appeared to link the tips of the microvilli with the outer, fibrillar layer of the yeast cell wall. Adhesion to the microvilli was followed by what appeared to be active destruction of the microvilli by the yeast cell. Extracellular phospholipases and proteinases produced by *C. albicans* have been suggested to play a role in this process (168). Typically, single yeast cells were found within epithelial cells enclosed in a non-membranous vacuolar space; unlike the phagocytosis observed by endothelial cells in which yeast were observed enclosed within a membranous vacuole (452). The cytoplasmic contents of the enterocytes did not appear to be disturbed by the yeast. Yeast then passed through the basal side of the enterocyte into the lamina propria where the yeast were either engulfed by phagocytic cells or formed microabscesses (8).

Penetrative growth through the epithelial lining by hyphae at the cardiac-atrium fold of the infant mouse stomach was a prominent feature of *C. albicans* GI invasion early after inoculation (76). In addition, colonized infant mice given a second insult by broad spectrum antibiotics, X-irradiation, or immunosuppressant agents, resulted in

overgrowth and hyphal penetration of the mucosa predominantly at the cardiac-atrium fold (74,150).

Translocation through the gut epithelium by the aid of M cells is also possible. A high frequency of *C. albicans* yeast translocation through the mucosal epithelium covering Peyer's patches of the small intestine was noted by Alexander and colleagues (8). Membranous (M) cells are interspersed in the follicle-associated epithelium overlying the Peyer's patches in the intestine, and continuously transport antigen by transcellular movement from the gut lumen to cells of the mucosal associated lymphoid tissue (MALT) (291). The MALT is a large, somewhat amorphous collection of mucosal immune system cells composed of cells contained in the Peyer's patches, lymphoid follicle cells, lamina propria and intraepithelial lymphocytes, and mesenteric lymph nodes. The basolateral surface of the M cell forms an intraepithelial pocket containing B and T lymphocytes and macrophages to which the M cells transport antigens. In GI translocation kinetic studies, orally inoculated latex beads or *Saccharomyces cerevisiae* initially associated with Peyer's patch enterocytes of the small intestine, presumably M cells (27,178,222). Yeast cells were not seen adhering to adjacent villi or crypts. The beads and yeast were then found within intraepithelial pocket macrophages, the intraepithelial space itself and phagocytes beneath the basalateral membrane. Phagocytic cells, notably macrophages from the germinal centers of Peyer's patches, transported the particles to the mesenteric lymph nodes (8). In an animal burn model, Inoue and colleagues observed *C. albicans* yeast cells within the mesenteric lymph nodes after dissemination from the GI tract (170). Therefore, it is highly possible that M cells transport *C. albicans* across the intestinal mucosa.

Leukocyte Chemoattractant Receptors

As stated previously, the main focus of my research was investigating the recognition of *C. albicans* by human phagocytic cells, particularly PMNs. As shown in the section, "Host Defense Mechanisms against *Candida albicans*," PMNs function as the primary effector cells against disseminated candidiasis. This is highly dependent upon the PMNs ability to migrate from the blood stream into the tissues where *C. albicans* has invaded. Once within the tissues, PMNs display chemotactic activity toward the foreign microorganism. Chemotaxis is the directed migration of cells along a chemical gradient. PMNs are attracted to the fungal cells by chemoattractants generated either directly from host cells in response to the fungal organism or due to the interaction of extracellular host factors with the fungus, e.g. fungal cell wall activation of the complement cascade. In addition, my research and experimental evidence from other researches, has shown that *C. albicans* produces a chemoattractant(s) for PMNs that does not depend upon host derived factors.

Human phagocytic leukocytes express a number of different chemoattractant receptors that recognize factors derived from both endogenous and exogenous sources. Classical chemoattractant factors include the anaphylatoxins C3a and C5a, *N*-formylated peptides (the best characterized being *f*Met-Leu-Phe (*f*MLF)), platelet-activating factor (PAF), and leukotriene B₄. Hence, the known receptors for these factors are termed the classical chemoattractant receptors: C5a receptor, C3a receptor (also known as AZ3B), formylated peptide receptor (FPR or FPR1), and PAF receptor (44,276). Furthermore, two other chemoattractant receptors expressed by leukocytes have recently been identified by their high homology to FPR, FPR like-1 receptor (FPRL1) and FPR like-2 receptor (FPRL2). In addition to the classical chemoattractants, the chemokines make up a large group of cytokines with chemotactic activity. There at least 17 human chemokine receptors and 36 chemokines known to date (453). The chemokines are divided into four

classes (α , β , δ and γ) based on sequence similarities and the position of four conserved cysteine residues (453). Each chemokine receptor has more than one chemokine ligand and individual chemokines generally bind to more than one receptor, making the relationships between the chemokines and their receptors very complex. All eukaryotic cell chemoattractant receptors are members of the 7-transmembrane domain receptor superfamily, and are coupled to pertussis toxin-sensitive guanine nucleotide-binding protein (G protein)-linked signalling pathways (307).

Bacteria release a heterogeneous mixture of small molecular weight *N*-formylated peptides that stimulate leukocyte chemotaxis (355,356). These peptides are present in nanomolar concentration in spent bacterial culture medium and display potent chemotactic activity for both human PMNs and macrophages (355). Characterization of the peptides in enriched butanol extracts from *Escherichia coli* culture supernatants showed that *f*MLF was the predominant peptide present responsible for PMN chemotactic activity (246). Separate experiments using a variety of synthetically produced NH₂-terminal blocked and unblocked peptides also showed *f*MLF to have the greatest activity of those tested ($K_d = 1$ nM) (126).

The expression of chemoattractant peptide receptors for formylpeptides was shown using radio- and fluorescently-labeled formylpeptides that bound to the plasma membrane of human (295,296) and rabbit (19) PMNs and a subpopulation of differentiated HL-60 cells (294). cDNA FPR clones were isolated using a strategy in which clones from a cDNA library constructed from granulocyte differentiated HL-60 cells conferred the binding of *N*-formylpeptides to transfected COS-7 cells (45). Subsequently, by using the same strategy and cross-hybridization under high stringency conditions FPRL1 and FPRL2 were identified (26,277,446). FPRL1 is 69% identical to FPR, but has ~1,000 fold lower affinity for *f*MLF ($K_d > 400$ nM) (277). Both FPR and FPRL1 are expressed in human PMNs and monocytes (101). Lipoxin A4, an arachidonic

acid derivative with anti-inflammatory functions, was suggested by Fiore and colleagues to be the ligand for FPRL1 (120), although others have not been able to reproduce this finding (44,385). A peptide derived from the ectodomain of human immunodeficiency virus type-1 gp41 was recently shown to be an agonist for FPRL1 (385). FPRL2, is 56% identical to FPR and 72% identical to FPRL1 (277,446). The ligand for FPRL2 is unknown. It does not recognize *N*-formylated peptides nor does it appear to be expressed in PMNs (101).

Standard ligands for FPR consist of bacterial and mitochondrial peptides that are synthesized with *N*-formylmethionine as the starting residue (59,239,246,348,355,356,361). Some nonformylated peptides have also been shown to stimulate FPR-mediated chemotaxis (126,386,397), but with the exception of a few cases (135), the chemotactic activity induced by these peptides was significantly lower than that of their formylated counterparts (126). Concentrations of ligand 10- to 100- fold greater than that required for chemotaxis stimulate anti-microbial and cytotoxic functions through FPR, such as the release of inflammatory granule contents and activation of superoxide production by the NADPH-oxidase (180). The role of FPR in host defense has been continually questioned because of the lack of direct evidence that phagocytes recognize bacterially derived *N*-formylated peptides *in vivo*.

The first *in vivo* evidence that FPR does function in host defense was obtained using FPR1 knock out mice (136). The mouse gene *Fpr1* encodes a receptor that is 77% identical to human FPR. The EC₅₀ for *Fpr1* is approximately 100-fold higher than for human FPR, as measured by intracellular calcium mobilization in response to *f*MLF (137). Therefore, mouse *Fpr1* is considered a low affinity receptor for *N*-formylated peptides. Although mice have not been shown to express a high affinity receptor for *N*-formylated peptides(137), the removal of *Fpr1* resulted in decreased resistance to *Listeria monocytogenes* injected by IV compared to *Fpr1*^{+/+} littermates (136). Interestingly,

heterozygous mice had an intermediate phenotype, suggesting a gene dosage effect in both *L. monocytogenes* IV challenge experiments and functional FPR assays *in vitro*. Neutrophilic abscesses were observed in liver and spleen of *Fpr1*^{-/-} mice, although there were increased numbers of *L. monocytogenes* CFUs in these organs versus *Fpr1*^{+/+} mice. The researchers suggested that FPR deficiency does not affect neutrophil trafficking into the organs but does affect the regulation of innate immunity.

I present evidence in this thesis that non-serum dependent chemotactic factors (NSCFs) released from *C. albicans* yeast stimulated human PMN chemotaxis by interacting with FPR. The recognition of *C. albicans* yeast derived NSCFs for guinea pig PMNs was first shown in 1977 by Jim E. Cutler (84). We showed that FPR-mediated chemotaxis accounted for approximately half of the PMN chemotaxis toward NSCF(s) in the *C. albicans* culture filtrate. In addition, Chinese hamster ovary (CHO) cells expressing FPR displayed chemotaxis toward *C. albicans* culture filtrates, while CHO-wild type cells did not. CHO-FPR chemotaxis toward *C. albicans* NSCF was inhibited with the FPR antagonist, *tert*-butoxycarbonyl-Met-Leu-Phe (*t*-Boc-MLF). These results provide another mechanism by which *C. albicans* may stimulate the host's inflammatory immune response.

C. albicans secreted aspartyl proteinase (SAP) was also found to be chemotactic, as well as chemokinetic, for human PMNs (405). Maximal chemotactic activity was at 500 nM and was greatly reduced by heating SAP at 100°C for 10 min. Intra-dermal injection of purified SAP induced PMN infiltration around the dermal vessel, suggesting that SAP displayed chemotactic activity *in vivo*.

Others have noted a correlation between virulence and decreased stimulation of PMN chemotaxis by specific *C. albicans* strains (416). However, in results reported here, *S. cerevisiae* (a species rarely implicated in disease) and all *Candida* spp. tested produced a NSCF for human PMNs. In addition to these findings, others have shown

that *Trichophyton mentagrophytes* and *Blastomyces dermatitidis* release low molecular weight chemotactic substances for PMNs (361,391,396). Therefore, a wide range of fungi can produce non-serum dependent PMN chemoattractants.

Furthermore, we demonstrated that the NSCF(s) attracts PMNs across an intestinal epithelial cell monolayer *in vitro*, suggesting that NSCF was active across a mucosal surface. We also provide evidence for an additional chemotactic agent produced by *C. albicans* that stimulated chemotaxis of the murine macrophage-like cell line, J774, by a different receptor. As already discussed, a high affinity FPR has not been shown to be expressed by murine leukocytes (137). J774 cell chemotaxis could not be stimulated by *f*MLF, nor was chemotaxis toward NSCF inhibited by *t*-Boc-MLF, suggesting that chemotaxis was stimulated through a different receptor by the same chemoattractant or a separate factor contained in the culture filtrate. Considering that the *C. albicans* NSCF contained in the culture filtrates stimulated human PMNs through FPR and J774 cells by a different receptor, it would be very interesting to determine the response of *Fpr1*^{-/-} mice to a *C. albicans* infection.

PMN β_2 -Integrins and Leukocyte Adhesion Deficiency-Type I

Integrins are a class of cell membrane glycoproteins made up of covalently bound $\alpha\beta$ heterodimers that are involved in cell adhesion functions. Currently, there are 8 known β subunits and 14 known α subunits (2). The formation of $\alpha\beta$ heterodimers is restricted, such that not all β subunits bind to all α subunits, and vice-versa. Therefore, integrins are further subdivided into groups based on the association of a particular β subunit with various α subunits (e.g. β_1 -integrins, β_2 -integrins, etc.).

Leukocyte adhesion deficiency-type 1 (LAD-1) is an autosomal recessive disorder due to a genetic defect in the gene ITGB2 that encodes CD18. CD18 is the 95 kDa glycoprotein, that is the β -subunit of β_2 -integrins (200,379). CD18 forms a heterodimer

with one of three α -subunits, CD11a, CD11b, CD11c, or CD11d (257,439). The β_2 -integrins are expressed in varying degrees by all human leukocytes and lymphocytes. They play a role in both cell-matrix and cell-cell adhesion functions. Other names for β_2 -integrins commonly used in the literature are: [1] CD11a/CD18; LFA-1, Leu CAMa, and $\alpha L/\beta_2$, [2] CD11b/CD18; complement receptor (CR) 3, Leu CAMb, Mac-1, Mo1, OKM-1, and $\alpha M/\beta_2$, [3] CD11c/CD18; p150 (p150, 95), CR4, Leu CAMc, and $\alpha X/\beta_2$, [4] CD11d/CD18; $\alpha D/\beta_2$ (2). To be consistent with other sections of this thesis, I will refer to CD11b/CD18 as CR3 and CD11c/CD18 as CR4.

CD18 deficiency results in the loss of function, and usually expression, of all β_2 -integrins (4,257). Although, patients that retain surface expression of non-functional β_2 -integrins have been described (164,213). In addition, a moderate phenotype is associated with patients that express low levels (1-10%) of functional β_2 -integrins (10,93). LAD-1 patients display recurrent bacterial infections, impaired pus formation and wound healing, and neutrophilia (408). Granulocytes (83), monocytes (242) and lymphocytes (93,256) all display a wide variety of adhesion-dependent function abnormalities. PMN defects are noted in respiratory burst regulation (367), chemotaxis (213,285) and phagocytosis (367). All are secondary effects of cell adhesion abnormalities. Lymphocyte functions are also severely impaired (185,353), although the pathology in LAD patients is primarily due to deficient leukocyte function. Severity of clinical infections among LAD patients directly correlated with the degree of expression of β_2 -integrins, such that patients with some expression of functional β_2 -integrins displayed less infectious complications and a longer life expectancy than those with no expression of β_2 -integrins (10). Patients with a complete loss of β_2 -integrins expression usually do not survive past the first year of life (398).

Another syndrome, LAD-type 2 (LAD-2), is a separate genetic disorder also characterized by leukocyte adhesion malfunctions (3,184). LAD-2 patients have a defect in the *de novo* pathway of GDP-fucose biosynthesis that results in aberrant glycosylation of some cell surface glycoproteins (184,384). Therefore, patients do not express the sialyl-Lewis X ligands for selectins that are necessary for the initial adhesion of PMNs to endothelium (109). LAD-2 patients also display recurrent bacterial infection, impaired pus formation, and impaired wound healing (109).

Restoration of β_2 -integrin expression and function is necessary to cure LAD-1 patients. Treatment for LAD-1 patients includes bone marrow transplantation (121,284) with or without gene therapy (435,448). Gene therapy was attempted by retrovirus-mediated transduction of a functional human CD18 gene into the bone marrow progenitor cells of a LAD patient (435,448). In addition, one LAD patient was treated with human recombinant IFN- γ (hrIFN- γ) to try to increase Fc γ RI (CD64)-mediated effector functions of leukocytes, primarily PMNs (423). Although the patient died after 10 weeks of treatment, due to surgical complication, the patient had not experienced any new infections or side-effects due to the rIFN- γ since treatment was initiated (423). This suggests that rIFN γ treatment may be a way to restore some phagocytic cell effector functions.

LAD animal models include a CD18-deficient mouse that displays 2-16% of normal β_2 -integrin levels on leukocytes (436), a CD18-null mouse with no expression of β_2 -integrins (353,419), and bovine LAD (BLAD) (186) with $\leq 2\%$ β_2 -integrin expression of normal PMNs (285,367). Almost all BLAD animals were produced from a naturally occurring point mutation in the gene for CD18 that occurred in a Holstein bull, Osborndale Ivanhoe (365). Due to this bull's "high genetic merit" for producing cows with superior milk production, he, several of his sons, and grandsons have sired thousands of cows. Because of the practice of inbreeding in the dairy industry, BLAD

has become the most common bovine genetic disease worldwide and a serious economic concern (365). Studies using these animals, as well as cells isolated from LAD patients, have proven invaluable in defining the role of β_2 -integrins in a multitude of adhesion-related functions of immune cells (83,213), interactions of β_2 -integrins with other cell surface receptors (257), and cell signaling pathways stimulated through β_2 -integrins (33,51).

Studies have implicated that β_2 -integrins are necessary for PMN transendothelial migration (171). For a review of the molecular mechanisms of PMN transendothelial migration, refer to the section "Host defense mechanisms against *Candida albicans*; emphasis on PMNs." Interestingly, when PMN transendothelial migration was evaluated in LAD animals, the role of β_2 -integrins was found to be dependent upon the type of endothelium. Impaired PMN extravasation into the peritoneal cavity elicited by thioglycollate injection was observed in CD18-deficient mice (436) and CD18-null mice (419). Although, in CD18-null mice generated by a different research group (268), PMN migration into the peritoneal cavity was actually greater than in the control animals. The reason for this discrepancy is unknown. PMN transmigration into the respiratory tract of CD18-null mice (268) and BLAD calves (5,6) occurred at the same rate as in control animals. These results suggest that PMN transendothelial migration may occur by a β_2 -integrin-independent mechanism depending upon the type of endothelium.

Interestingly, using LAD monocytes it was determined that transendothelial migration occurred by β_2 -integrin- and very late antigen-4 (VLA-4, $\alpha 1\beta 4$)-dependent mechanisms across non-activated HUVEC monolayers (67). Transendothelial migration across IL-1 α , TNF- α , or LPS pretreated monolayers, was not dependent upon β_2 -integrins and was completely inhibited by monoclonal antibodies (mAbs) against VLA-4. LAD patient monocytes displayed considerable VLA-4-dependent migration as well. Therefore, transendothelial migration of PMNs and monocytes may occur by β_2 -

independent mechanisms and monocyte migration of LAD patients may not be totally impaired.

β_2 -integrins have been linked to promoting apoptosis of PMNs (418). Enhanced onset of apoptosis was observed in PMNs after transendothelial migration and antibody cross-linking of CR3 expressed on TNF- α treated PMNs. Therefore, the neutrophilia observed in human LAD patients (257) and LAD animals (275,419) may be due to decreased apoptosis of PMNs together with heightened PMN production in response to microbial infections.

The profound effects on both innate (149,257,302,367) and acquired (256,353,400) immune functions observed in LAD patients results not only because of loss of the adhesive functions of β_2 -integrins themselves, but also due to the loss of interaction between β_2 -integrins and other surface adhesins expressed on the immune cells (399). LAD PMNs displayed altered expression of various receptors. CR1 (CD35) expression on non-activated BLAD PMNs was about the same as normal control PMNs, yet expression was 2-fold greater on normal PMNs versus BLAD PMNs when cells were activated with PMA (440). L-selectin was reduced on BLAD PMNs compared to normal PMNs (420). Unstimulated LAD PMNs had enhanced expression of all three Fc γ receptors, Fc γ RI (241), Fc γ RIIa (440) and Fc γ RIIIb (281,283,440) compared to normal PMNs. Even though there is increased expression of Fc γ Rs on LAD PMNs, LAD patients displayed impaired antibody (IgG)-dependent phagocytosis and cytolysis (214,242,287). CR3 and CR4 have been found to cooperate with Fc receptors to mediate Ab-dependent effector functions (399). Cooperation between Fc receptors and β_2 -integrins on PMNs resulted in increased adhesion and anti-microbial responses, such as enhanced respiratory burst (215,450). Additionally, linking of CR3 to soluble Fc γ RIIIb can induce the release of proinflammatory cytokines from monocytes and PMNs (134). Therefore, augmentation of the majority of PMN phagocytic functions at inflammatory

sites are found to be absolutely dependent upon CR3 (149).

The β_2 -integrins have been shown to act as signaling partners on PMNs for a variety of receptors by interacting in a cis-acting fashion (37,399). CR3 has been shown to associate with and transduce cellular signals for the GPI-anchored receptors Fc γ RIIIb (CD16), urokinase-type plasminogen activator receptor (uPAR, CD87), and LPS receptor (CD14) that binds LPS/LPS-binding protein (LBP) complex (399). CR4 has also been shown to bind uPAR and uPAR appears to shuttle between CR3 and CR4 during chemotaxis (37). The LPS portion of the LPS/LBP complex bound to CD14 is believed to be responsible for binding to CR3 (399). In addition, Fc γ RIIa (CD32a), a single transmembrane protein (407), associates with CR3 by binding to CD11b, but does not bind to CR4 (13,441). Ligation of CR3 with anti-CR3 mAbs was sufficient to induce Fc γ RIIa association with the actin cytoskeleton (450). Phosphorylation of the cytoplasmic tail of Fc γ RIIa was induced when CR3 and Fc γ RIIIb were cross-linked together (450), and phosphorylation of Fc γ RIIa was found to be necessary for stimulating a respiratory burst through CR3-Fc γ RIIIb. Direct ligation of Fc γ RIIa resulted in a respiratory burst without CR3-Fc γ RIIIb activation. Ligation of CR3-Fc γ RIIIb or Fc γ RIIIa are thought to stimulate O $_2^{\bullet-}$ production through separate cell signalling cascades (451). Additionally, Fc ϵ R (CD23), also a single transmembrane protein, expressed by monocytes and B cells (197), interacts specifically with both CR3 and CR4 through their α -subunits, resulting in the production of reactive nitric oxide species and pro-inflammatory cytokines (220).

β_2 -integrins not only provide a means for cell signalling for the receptors discussed above, but also a link to the cytoskeleton. Co-capping experiments suggested direct-physical linkage between CR3 and Fc γ RIIIb, uPAR (also with CR4), and CD14 (399). A transmembrane link to the cytoskeleton is necessary for capping membrane proteins. CR3-Fc γ RIIIb and CR3-uPAR co-capping was inhibited with the

carbohydrates α -methylmannoside, D-mannose, and N-acetyl-D-glucosamine, suggesting that a lectin-carbohydrate interaction may be responsible for mediating the association (399). Therefore, β_2 -integrins promote the redistribution of membrane components on the surface of PMNs, a process that is extremely important for cellular functions, such as chemotaxis. For example, uPAR expression is localized to the leading edge of migrating inflammatory and neoplastic cells, where it focuses the proteolytic activity of uPA to convert plasminogen (bound to adjacent receptors) to plasmin that, in turn, degrades ECM proteins in the path of the cell (37,399). LAD PMNs were found to be deficient in capping of these three receptors (198), although patching of these receptors was not defective, suggesting the mobility of these receptors was not impaired.

No evidence exists for a *cis*-acting function between β_2 -integrins and Fc γ RI (CD64). Fc γ RI, the high affinity receptor for monomeric Fc γ (314,339), is a single transmembrane protein. PMNs do not normally express Fc γ RI, but unstimulated LAD PMNs show enhanced expression of Fc γ RI (241). Fc γ RI expression on normal PMNs can be up-regulated with IFN- γ and granulocyte-macrophage-colony stimulating factor (314,339), and was also shown to be upregulated *in vivo* during bacterial sepsis (366). LAD PMNs, but not normal PMNs, displayed enhanced chemiluminescence, representing superoxide production, in response to murine IgG2a-opsonized sheep RBCs (IgG2a-SRBCs) and phagocytosis of IgG2a-SRBCs (241). Both of these responses were inhibited by monomeric human IgG, suggesting they were Fc γ RI-mediated (241). Using LAD PMNs, it was determined that β_2 -integrins and Fc γ Rs, Fc γ RIIa and Fc γ RIIIb, were required for mAb-mediated anti-tumor PMN ADCC (214). Additionally, LAD monocytes displayed ADCC by a Fc γ RI-mediated fashion, but not by Fc γ RIIa (242). Therefore, cell effector functions mediated by Fc γ RI are not thought to be dependent upon CR3 and may be enhanced in LAD patients.

PMNs are thought to express two distinct C1q receptors, one that triggers superoxide production (C1qR_{O₂}) and one that up-regulates phagocytosis (C1qR_P) (394). C1q triggers superoxide production in a CD18-dependent manner (144), as shown by the complete unresponsiveness of LAD PMNs to C1q and inhibition of superoxide production from normal PMNs by anti-CD11b or CD18 mAbs. Adhesion of PMNs to a surface coated with C1q was inhibited by these mAbs as well. How CR3 interacts with C1qR_{O₂} is unknown.

Intracellular Ca²⁺ release stimulated by a variety of stimulants has been evaluated in LAD PMNs. Ca²⁺ acts as a secondary messenger within the cell. A decreased Ca²⁺ response was observed when LAD PMNs were treated with serum-opsonized zymosan (282), Concanavalin A (282,287), and bovine serum albumin-IgG immune complexes (359). LAD PMNs displayed a slight decrease, but longer lasting Ca²⁺ response to heat aggregated-IgG than control PMNs (282,287). LAD PMN response to PMA, a potent protein kinase C activator, was identical to normal PMNs (282). Therefore, β₂-integrins interact with a variety of surface receptors, but not all, to trigger intracellular signalling responses that result in effector functions.

Superoxide (O₂^{•-}) production by the NADPH-oxidase is an important effector function of PMNs. The production of O₂^{•-} is dependent upon the stimulus, and a number of studies have evaluated the ability of LAD PMNs to mount a sufficient respiratory burst. In general, a decreased or absent respiratory burst is observed when LAD PMNs are stimulated with serum-opsonized zymosan (283,287), IgG-opsonized *S. cerevisiae* (216), surface bound C1q (144), and a combination of anti-CR3 and anti-FcγRIIIb mAbs (450). LAD PMN O₂^{•-} production was the same as control PMNs when stimulated with Concanavalin A (283), heat aggregated-IgG (283,287), IgG-SRBCs (283), and anti-FcγRIIIa mAb (450). An enhanced superoxide response was observed when LAD PMNs were stimulated with mIgG2a-SRBCs (241) and PMA (367). Some have noted the O₂^{•-}

response to PMA was not enhanced in LAD PMNs, but instead sustained longer than normal PMNs (216). Therefore, superoxide functions might be impaired depending upon the stimulus and β_2 -integrins appear to serve a regulatory role in $O_2^{\bullet-}$ production.

CHAPTER 2

NON-SERUM DEPENDENT CHEMOTACTIC FACTORS PRODUCED BY *CANDIDA ALBICANS*
STIMULATE CHEMOTAXIS BY BINDING TO THE FORMYL PEPTIDE RECEPTOR ON
NEUTROPHILS AND AN UNKNOWN RECEPTOR ON MACROPHAGESIntroduction

The number of systemic *Candida* infections continues to increase among humans due to factors such as immunosuppressive therapeutic regimes, long-term catheterization, broad-spectrum antibiotic use, and increased survival time of immunologically compromised individuals (251,348,437). The gastrointestinal (GI) tract is believed to be one site of entry for *C. albicans* into the blood stream of immunocompromised individuals (73,188). *C. albicans* is commonly found as a commensal in the GI tract of humans (73) but dissemination from this site is uncommon without immunosuppression, such as suppression of the GI bacterial normal flora and neutropenia (188). The containment of *Candida* spp. to the GI mucosa as a commensal in immunologically normal individuals is not fully understood (76,188,297,298).

Both non-specific and specific immune defenses play a role in protection against disseminated candidiasis. Polymorphonuclear leukocytes (PMNs) have been shown to be the primary components of the host's innate immune defenses against disseminated candidiasis in *in vitro* studies, animal models, and neutropenic patient studies (90). A protective role for macrophages in disseminated candidiasis has also been suggested (23,35,325). In a murine model, promotion of a Th type-1 cell response by the release of IL-12 from professional phagocytic cells has been correlated with resistance to systemic candidiasis (336). Additionally, antibodies have been shown to play a protective role against systemic and vaginal candidiasis (61,153,252,253) and may act by enhancing the activity of professional phagocytic cells (54,154). Thus, a better understanding of the interactions between *C. albicans* and professional phagocytic cells would provide

valuable insights into how the body protects itself against this opportunistic pathogen. In particular, it is important to further characterize factors released from *C. albicans* that are recognized by phagocytic cells and attract them toward the site of infection or colonization.

In this chapter, we describe the production of a culture filtrate containing non-serum dependent chemotactic factors (NSCFs) from a variety of *Candida* spp. yeast forms and *Saccharomyces cerevisiae*. A *C. albicans* culture filtrate induced the attraction of both human PMNs and the murine macrophage-like cell line J774. Using an in vitro model of PMN migration across the intestinal epithelium (310,311) (simulating the transmigration of PMNs into the GI lumen), we have observed transmigration of PMNs toward culture filtrates containing the NSCF. We also determined that formyl peptide receptor (FPR)-mediated chemotaxis of human PMNs was responsible for a significant portion of the observed PMN chemotaxis. The results presented in this study suggest that there are at least two NSCFs produced by *C. albicans*. One NSCF attracts macrophages and the other PMNs.

Materials and Methods

Mammalian Cell Culture

T84 epithelial cells were purchased from American Type Culture Collection. J774, clone number 8, and wild type Chinese hamster ovary (CHO) cells were a kind gift from Dr. Ira Mellman, Yale University, New Haven, CT. T84 epithelial cell monolayers were grown in tissue culture dishes and on polycarbonate permeable supports with a surface area of 0.33 cm² (63,311). Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagles' medium (DMEM) and Ham's F-12 medium supplemented with 50 U/ml penicillin, 0.05 mg/ml streptomycin, 5% fetal bovine serum (FBS), and 15 mM HEPES. Wild type CHO cells and CHO cells transfected with the human FPR (CHO-

FPR) (267) were grown as adherent monolayers in α -modified minimum essential medium eagle (α -MEM) supplemented with 50 U/ml penicillin, 0.05 mg/ml streptomycin, and 5% FBS. J774 cells were grown in spinner flasks (Bellco Glass, Inc., Vineland, NJ) in DMEM containing, 50 U/ml penicillin, 0.05 mg/ml streptomycin, and 5% FBS.

Isolation of Human PMNs

Normal human PMNs were isolated from non-coagulated citrated blood by a gelatin sedimentation technique which gave approximately 90% pure PMNs as determined by microscopic evaluation (311). PMNs were suspended at a concentration of 5×10^7 cells/ml, not counting mononuclear cells, in modified Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, MO) containing (in g/l) 0.185 CaCl_2 , 0.098 MgSO_4 , 0.4 KCl, 0.06 KH_2PO_4 , 8 NaCl, 0.048 Na_2HPO_4 , 0.01 glucose, and 10 mM HEPES (pH 7.4, 4°C) (HBSS(+)). PMNs were kept on ice, up to 2 h until use.

Yeast

All yeast strains were from the stock collection at Montana State University and included *C. albicans* strains CA-1, A9, 105, 222a, LGH1095Y, and ATCC64550. Other yeast species in this study included *C. lusitaniae* (ATCC 64125), *C. parapsilosis* (ATCC 90018), *C. tropicalis* (ATCC 750), *C. glabrata* (ATCC 90030), and *Saccharomyces cerevisiae* (2180WT). Identification of all strains was confirmed with API 20C yeast identification strips (Analytab Products, Plainview, NY). All isolates were cultured from glycerol stock cultures held at -70°C and plated onto Sabouraud dextrose agar (SDA) (Difco Laboratories, Detroit, MI) for 48 h at 37°C . *C. albicans* strain CA-1 was used in all experiments except where otherwise noted. This strain was originally characterized by Hasenclever's original antiserum as a serotype A strain (160,181). However, by the Candida Check system (Iatron Laboratories Inc, Tokyo, Japan), CA-1 was a serotype B

strain. Others have noted discrepancies between the two methods of serotyping (153).

Production of Non-Serum Dependent Chemotactic Factor (NSCF) by Yeast Isolates

Culture filtrates containing NSCF were prepared the same way for all yeast isolates. A single yeast colony from a Sabouraud dextrose agar plate was inoculated into glucose (2%), yeast extract (0.3%), peptone (1%) broth (GYEPB), incubated to stationary phase at 37°C for 24 h with aeration, and subcultured to fresh 2% GYEPB and incubated to stationary phase. Yeast cells were then harvested by centrifugation, washed 3x in HBSS(+), and suspended in HBSS(+) at 5×10^8 cells/ml or other cell concentrations as indicated. Yeast suspensions were incubated for various times at 37°C under vigorous aeration by rotation at approximately 200 rpm (Controlled Environment Incubator Shaker model M52 New Brunswick Scientific Co., Inc. Edison, NJ). Yeast cells were removed by centrifugation and the culture supernatant containing the NSCF was filtered through a sterile 0.2 μm cellulose acetate filter (Costar, Cambridge, MA). Culture filtrates were kept on ice or stored at 4°C until use. No loss of chemotactic activity was observed for culture filtrates stored up to 1 month. For filtration experiments, 1 kDa and 0.5 kDa cut off filters (Amicon Inc., Beverly, MA) were used.

PMN Transmigration Across an Epithelial Monolayer

For transmigration experiments, T84 intestinal epithelial cell monolayers were grown in cell culture inserts on permeable polycarbonate filters (Corning Costar, Inc., Cambridge, MA) with 5.0 μm pores (310). T84 monolayers were cultured both in the standard (apical surface upward) configuration and in the inverted configuration (basolateral surface upward) to permit transepithelial migration in the apical-to-basolateral and basolateral-to-apical directions. For inverted monolayers, the porous

supports were fitted with a 0.8-mm thick lexan ring with the same dimensions as the base of the Costar insert (Harvard University Machine Shop, Boston, MA). The ring was attached to the underside of the insert using General Electric RTC Silicone (no. 108, translucent) glue making sure to not apply any glue to the porous support itself. The glue was allowed to dry over night at room temperature. Inserts were then sterilized by submersion in 70% ethanol, inverted onto a sterile petri dish in a hood, and allowed to dry. Rat tail collagen (88) was then applied to the underside of the porous support. Cells were applied to the underside of the porous support and allowed to attach overnight before righting the inserts into the 24-well holding plates. The confluence and tight junction formation of monolayers were determined by measuring the transepithelial resistance with an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) (239). Prior to addition of PMNs, monolayers were washed extensively with HBSS(+) to remove the tissue culture medium containing FBS. For apical-to-basolateral transmigration experiments, 2×10^6 PMNs were added to the top chamber and allowed to transmigrate into the lower well containing either $1 \mu\text{M}$ *f*-Met-Leu-Phe (*f*-MLF) (as a positive control) or the yeast culture filtrate. In a subset of experiments, transmigration was performed with T84 monolayers in the standard configuration after treatment of monolayers with 2 mM EDTA for 12 min at 37°C (309), to disrupt the tight junctions. Such experiments were performed exactly as the standard apical-to-basolateral assays except that half the concentration of PMNs was added. For basolateral-to-apical transmigration experiments, 1×10^6 PMNs were added to the top well and allowed to transmigrate into the lower well containing either 100 nM *f*-MLF (as a positive control), or the yeast culture filtrate. After incubating for 110 min at 37°C , the transmigrated cells and cells contained within the T84 cell monolayer were quantified by a myeloperoxidase assay (311).

Chemotaxis Across a Membrane Filter

Cell culture inserts with 5.0 and 8.0 μm diameter pore sizes were used for chemotaxis of PMN and CHO or J774 cells, respectively. PMNs (10^6) were suspended in HBSS(+) for chemotaxis assays or in culture filtrate or *f*-MLF (10 nM) for chemokinetic experiments and directly placed in the upper well of the filter insert. For some experiments, PMNs were treated with *t*-butoxycarbonyl-Phe-Leu-Phe-Leu-Phe (*t*Boc-FLFLF) (33 μM final concentration) or dimethyl sulfoxide (DMSO) (0.3% [v/v] final concentration) for 10 min at 4°C before being added to the upper well. PMNs migrated into the lower well containing either 10 nM *f*-MLF (as a positive control) or the yeast culture filtrate. After 110 min at 37°C, the number of PMNs in the lower well was determined by the myeloperoxidase assay. Results were normalized by setting the average value for *f*-MLF-induced chemotaxis to 100.

At 14-16 h before use in the chemotaxis experiments, 6 mM (final concentration) Na-butyrate was added to the adherent CHO cells to increase cellular protein expression (146). The CHO cells were then harvested from the tissue culture plate surface with 1x trypsin-EDTA (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline without Ca^{2+} and Mg^{2+} and suspended in α -MEM containing FBS (5%) for 1 h at 37°C. CHO cells were washed 2x with serum-free α -MEM containing 10 mM HEPES (pH 7.4) and suspended to 2×10^6 cell/ml in serum-free α -MEM containing 10 mM HEPES (pH 7.4). For J774 cell chemotaxis experiments, the cells were removed from the spinner flasks, washed 2x with serum-free DMEM containing 10 mM HEPES (pH 7.4) and suspended to 2×10^6 /ml in serum-free DMEM containing 10 mM HEPES (pH 7.4). For both CHO and J774 cell chemotaxis experiments, 3×10^5 cells (150 μl) were placed in the upper well and yeast culture filtrate or controls in the lower well. For some experiments CHO-WT and CHO-FPR cells were treated with *t*Boc-Met-Leu-Phe (*t*Boc-MLF) (33 μM final concentration) or DMSO (0.3% [v/v] final concentration) for 10 min

before addition to the upper well. As a positive fibroblast migration control, fibronectin (20 µg/ml) was used for both CHO-WT and CHO-FPR cells (20,258,258). In chemotaxis experiments, 1 nM *f*-MLF served as the positive control for CHO-FPR cells. Zymosan A (Sigma Chemical Co., St. Louis, MO) complement-activated human serum was used as the positive control for J774 cell chemotaxis. Zymosan A (1 mg/ml) was added to serum (30 min, 37°C), pelleted from solution and the supernatant was used as the positive control. CHO and J774 cell migration was carried out for 4 h at 37°C, after which time the filters were fixed with 2.5% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) for 2 h at room temperature or overnight at 4°C. The cells on the upper side were removed from the filter with a cotton swab and those that had migrated to the under side were stained with hematoxylin stain (Sigma Chemical Co., St. Louis, MO). After being stained, the upper side of the filter was again swabbed with cotton. For analysis, the filter was removed from the plastic holder. Quantification of cells was performed using a computerized image analysis system (Imaging Research M4 True Color Image Analysis System, Imaging Research, St. Catherines, Ontario) to determine the average cell area (µm²) per field from an average of 10 randomly chosen (avoiding the periphery) 40x fields. Results are equalized by setting the average value for chemotaxis induced by *f*-MLF or zymosan A activated serum to 100.

Results

Production of the Non-Serum Dependent Chemotactic Factor (NSCF) from *C. albicans* Yeast.

Samples of culture filtrates were taken at various times from HBSS(+) cultures inoculated with either 2×10^7 or 5×10^8 yeast/ml. As shown in figure 1, production of chemotactic activity in the cultures filtrate was time dependent. Chemotactic activity consistently peaked at 1 to 3 h for culture filtrates from the 5×10^8 yeast/ml culture.

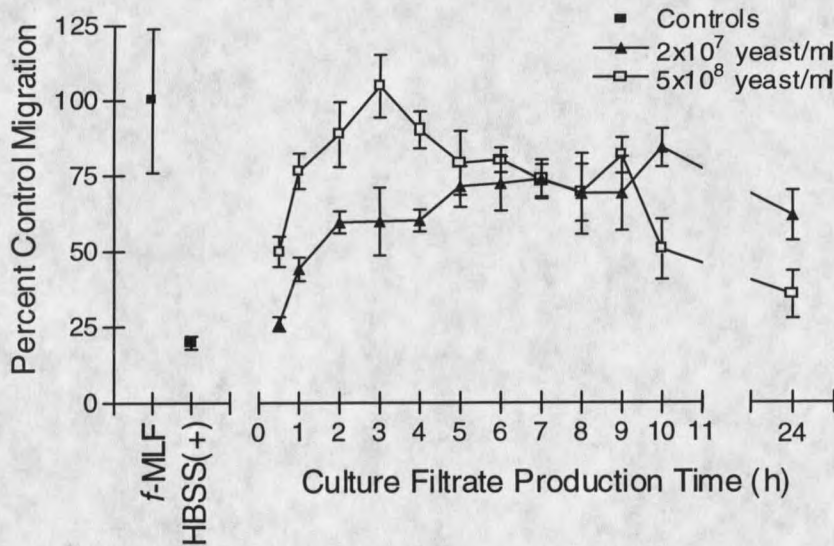


Figure 1. Production of NSCF by *C. albicans* is Time and Yeast Concentration Dependent. Chemotaxis of PMNs toward *C. albicans* culture filtrates produced with 2×10^7 yeast/ml (▲) or 5×10^8 yeast/ml (□) for 0.5 h to 24 h. PMN chemotaxis was assessed by quantification of the total number of PMNs found in the lower reservoir by a myeloperoxidase assay. HBSS(+) was used as a negative control and *f*-MLF (10 nM) as a positive control. Data points are of a representative experiment. All samples were run in triplicate. Error bars indicate SD.

Production of the chemotactic activity from the 2×10^7 yeast/ml culture was more delayed. The activity in the 2×10^7 yeast/ml culture leveled out at later time points and remained consistently high compared to the 5×10^8 yeast/ml culture, which showed a steady decrease after the early activity peak. The chemotactic activity in the cultures at the 24-h time point differed dramatically. In the high yeast concentration culture, the activity had dropped to almost the level of the negative control at 24 h, whereas the activity in the culture produced with a low concentration of yeast remained high at 24 h.

To confirm that the culture filtrate contained a chemotactic factor rather than a chemokinetic factor, a checkerboard analysis was performed (Table 1). PMN migration was found to be maximal when undiluted culture filtrate was used. With the addition of culture filtrate into the upper well, a decrease in migration was observed, suggesting that

Table 1. Checkerboard assay of PMN chemotaxis toward *C. albicans* culture filtrate^a

		Culture filtrate concentration in upper well				
		0	1:8	1:4	1:2	Undil.
Culture filtrate concentration in lower well	0	1.7 ± 0.5	1.2 ± 0.2	1.4 ± 0.3	2.3 ± 0.6	1.8 ± 0.3
	1:8	3.7 ± 0.4	1.3 ± 0.2	1.8 ± 0.3	1.5 ± 0.2	1.7 ± 0.3
	1:4	6.2 ± 0.6	1.5 ± 0.3	1.1 ± 0.2	2.9 ± 0.8	2.8 ± 0.6
	1:2	19.1 ± 0.7	3.7 ± 0.7	3.5 ± 0.5	7.0 ± 0.9	7.7 ± 0.8
	Undil.	32.9 ± 0.6	15.6 ± 2.7	12.7 ± 3.0	22.7 ± 1.2	14.3 ± 0.8

f-MLF induced PMN chemotaxis			
		f-MLF concentration in upper well	
		0	10 nM
f-MLF concentration in lower well	0	1.7 ± 0.5	1.2 ^b
	10 nM	12.4 ± 0.8	4.7 ^b

^a *C. albicans* culture filtrate produced by 5×10^8 yeast/ml for 1 h. Samples were diluted with HBSS(+). PMN chemotaxis was assessed by quantification of the total number of PMNs found in the lower reservoir by a myeloperoxidase assay. Results are the number of PMNs migrated ($\times 10^4$). Results are the average of samples run in triplicate \pm SD.

^b Samples run in duplicate only.

the activity was mostly due to chemotaxis, rather than chemokinesis (Table 1). Similar results were obtained when PMNs were allowed to migrate toward *f*-MLF in the presence or absence of *f*-MLF in the upper well (Table 1). These results suggest that the *C. albicans* culture filtrate stimulated PMN chemotaxis and contained chemokinetic activity at a level similar to that of 10 μ M *f*-MLF.

Characterization of the NSCF

NSCF stability, size range and culture parameters were determined (Table 2). Culture filtrate from a 1-h culture retained NSCF activity for over 1 month at 4°C. NSCF activity was lost following filtration of active culture filtrates through a cellulose acetate filter with a glass pre-filter, presumably due to adherence to the glass filter (data not shown). NSCF activity in the culture filtrate was not retained by a 1-kDa cutoff filter, and only partial activity for PMNs was recovered in the retentate when a 0.5-kDa cutoff filter was used (Table 2). These results suggest that the size of the NSCF is a small molecule with an apparent molecular weight between 0.5 and 1 kDa. The chemotactic activity of *C. albicans* NSCF was concentration dependent (Table 1). Dilution analyses of culture filtrates made with 5×10^8 yeast/ml for 0.5 and 1 h revealed that the chemotactic activity decreased to that of the negative control at a greater than eight fold dilution (data not shown). It was also determined that glucose was not required for the production of NSCF. However, no activity was produced when yeast cultures were produced without Ca^{2+} and Mg^{2+} (Table 2). Addition of the divalent cations to the culture filtrate did not restore NSCF activity (data not shown).

To examine whether the production of the NSCF was strain- or species-dependent, we tested the production of the factor by six different strains of *C. albicans*, a strain of *S. cerevisiae* and a variety of *Candida* spp. (Fig. 2). For all strains and species, 1-h culture filtrates stimulated PMN chemotaxis significantly more than the negative

Table 2. NSCF Characteristics^a

Characteristic	Conditions	PMN Chemotaxis	J774 Chemotaxis
Stability ^b	4°C, ≥ 1 mo.	Yes	Yes
	56°C, 30 min	Yes	Yes
	Boiling, 10 min	No	Yes
Size ^c	1 kDa retentate	21.9 ± 6.1	22.5 ± 14.8
	1 kDa filtrate	95.2 ± 27.6	119.7 ± 18.0
	0.5 kDa retentate	37.0 ± 5.0	ND
	0.5 kDa filtrate	59.8 ± 21.8	ND
Production Requirements ^d	Glucose	Chemotactic	Chemotactic
	Divalent Cations	Not chemotactic	Chemotactic

^a Characterization of the NSCF in a *C. albicans* 1 h culture filtrate. PMN chemotaxis was assessed by quantification of the total number of PMNs found in the lower reservoir by a myeloperoxidase assay.

^b Culture filtrate was held at 4°C (≥ 1 mo.), 56°C (30 min), and boiling (10 min) and then tested for PMN chemotactic activity.

^c Culture filtrate was passed through 1 and 0.5 kDa cut off filters. Retentate was diluted to the original volume with HBSS(+) to avoid concentration effects and then tested for PMN chemotactic activity. Values given are percentages of activity retained in the retentate compared to the non-filtered 1 h culture filtrate sample value that was set to 100. Migration of PMNs toward HBSS(+) was 14.2 ± 6.8. Migration of J774 cells toward HBSS(+) was 15.5 ± 5.3.

^d A *C. albicans* 1 h culture filtrate was produced using HBSS(+) without glucose and HBSS(-). Divalent cations were added back to the HBSS(-) culture filtrate (0.185 g/l CaCl₂ and 0.098 g/l MgSO₄) before determination of PMN chemotaxis.

control (Student's *t* test; *P* values ranged from 0.003 to <0.0001). Chemotaxis differences observed figure 2 are possibly due to PMN donor variability. Differences in the degree of chemotaxis toward the same chemoattractants by different donors' PMNs were routinely observed.

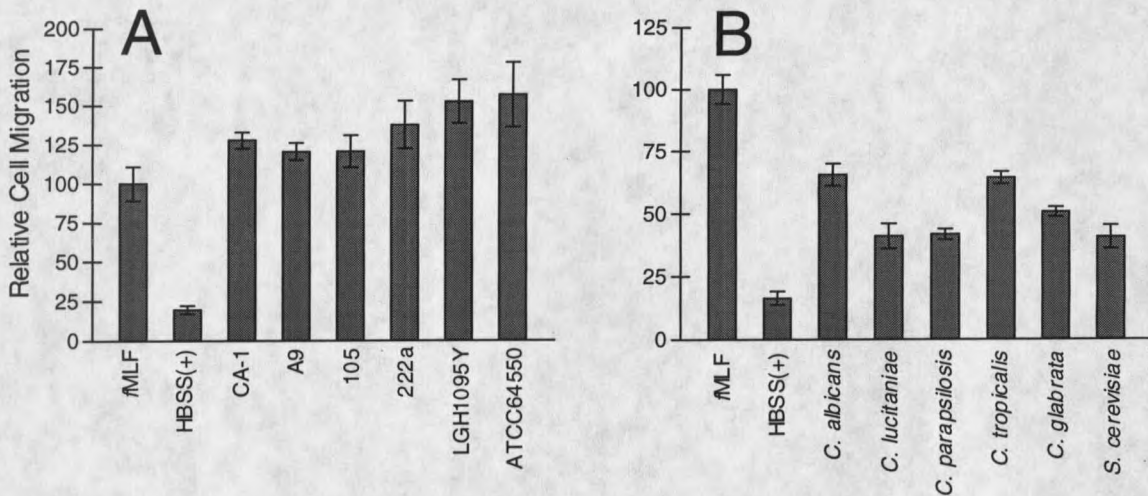
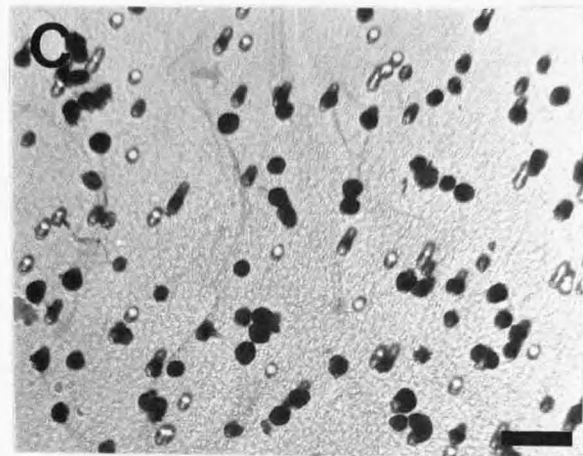
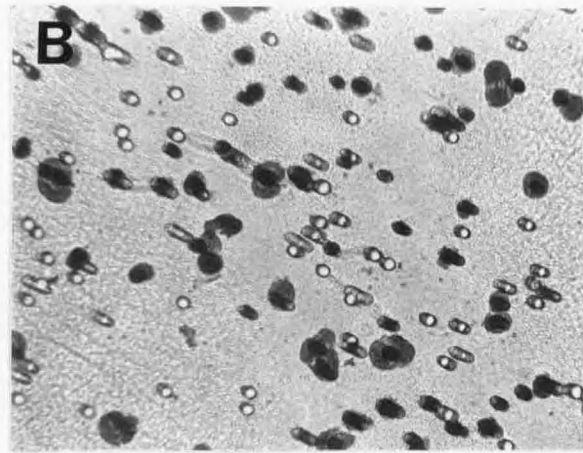
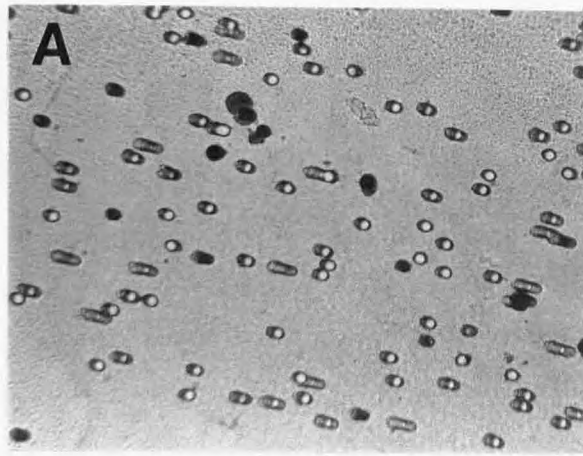


Figure 2. NSCF Production is Not Species or Strain Dependent. (A) Chemotaxis of PMNs toward 1 h culture filtrates produced by 6 different *C. albicans* strains. (B) Chemotaxis of PMNs toward 1 h culture filtrates produced by various *Candida* spp. and *S. cerevisiae* yeast forms. *C. albicans* in B is strain CA-1. Chemotaxis was assessed by quantification of the total number of PMNs found in the lower reservoir by a myeloperoxidase assay. The data are expressed as the mean \pm SEM from three different experiments with samples run in triplicate. Data points were equalized by setting the migration toward *f*-MLF (10 nM) to 100.

C. albicans NSCF Stimulates Chemotaxis by Binding to the Formyl Peptide Receptor

We previously generated a CHO cell line stably-expressing the human formyl peptide receptor (FPR) (CHO-FPR) and showed that these cells migrated toward a gradient of formylated peptides (266). Our preliminary characterization revealed that the NSCF appeared to be similar in size to formylated peptides, thus we performed experiments to determine if the NSCF could stimulate CHO-FPR chemotaxis. As seen in figure 3C and figure 4A, CHO-FPR cells displayed chemotaxis toward the 1-h culture filtrate, whereas CHO-WT cells did not (Fig. 4A). Both CHO-FPR and CHO-WT cells displayed chemotaxis towards fibronectin (20 μ g/ml), a natural chemoattractant for fibroblasts (20,258) (Fig. 4A). Chemotaxis of CHO-FPR cells towards the culture filtrate was inhibited by the FPR antagonist *t*Boc-MLF, confirming that the CHO-FPR cell

Figure 3. Migration of CHO-FPR Cells Toward *C. albicans* 1 h Culture Filtrate. CHO-FPR cells migrated through 8.0 μm semi-porous supports for 4 h, 37°C, toward (A) HBSS(+), (B) *f*-MLF (1 nM), and (C) 1 h culture filtrate. CHO-FPR cells adhered to the underside of the support were stained with hematoxylin. Bar, 50 μm . (Student's *t*-test, *P* values ranged from 0.003 to <0.0001).



chemotaxis was mediated by FPR. In addition, the FPR antagonist *t*Boc-FLFLF inhibited the chemotaxis of PMNs toward the 1-h culture filtrate by approximately 51% (Fig. 4B).

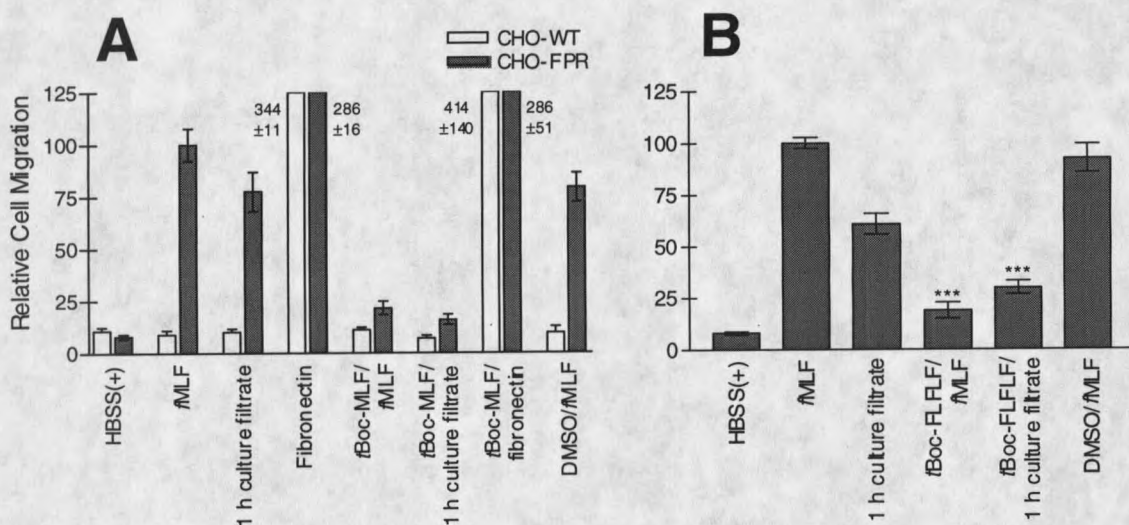


Figure 4. *C. albicans* Culture Filtrate Contains a Chemotactic Factor That Activates Chemotaxis Through FPR. (A) Wild type CHO cells (open bars) and CHO cells expressing FPR (filled bars) or (B) PMNs were stimulated to migrate toward *C. albicans* 1 h culture filtrate or *f*-MLF (1 nM). Fibronectin (0.02 mg/ml) was used as a positive control for chemotaxis of CHO cells. Some CHO cells and PMNs were preincubated with the FPR antagonists, *t*Boc-MLF (10 μ M, 10 min, 37°C), or *t*Boc-FLFLF (10 μ M, 10 min, 4°C), respectively, before being placed in the upper well. The final concentration of DMSO in the wells containing agonist was 33 μ M. This concentration of DMSO by itself did not significantly decrease chemoattractant-induced migration. (A) Chemotaxis of CHO cells was assessed by quantification of the average cell area of hematoxylin stained cells that had migrated and adhered to the underside of the porous support. A total of 10 randomly chosen 40x fields were examined. The data are expressed as the mean \pm SEM from three different experiments. Data points were equalized by setting the migration of CHO-FPR cells toward *f*-MLF to 100. (B) Chemotaxis of PMNs was assessed by quantification of the total number of PMNs found in the lower reservoir by a myeloperoxidase assay. The data are expressed as the mean \pm SEM from three different experiments with samples run in triplicate per experiment. Data points were equalized by setting the migration toward *f*-MLF (10 nM) to 100. *** Student's *t* test, $P = 0.0001$.

PMN Transmigration Across a Cultured Intestinal Epithelial Monolayer

Since the GI tract is considered to be a major portal of entry to the blood stream, we tested whether the culture filtrate could attract PMNs across a monolayer of T84 intestinal epithelial cells grown on permeable supports (309). As shown in figure 5A, the filtrate from a 1-h *C. albicans* culture stimulated basolateral to apical migration of PMNs.

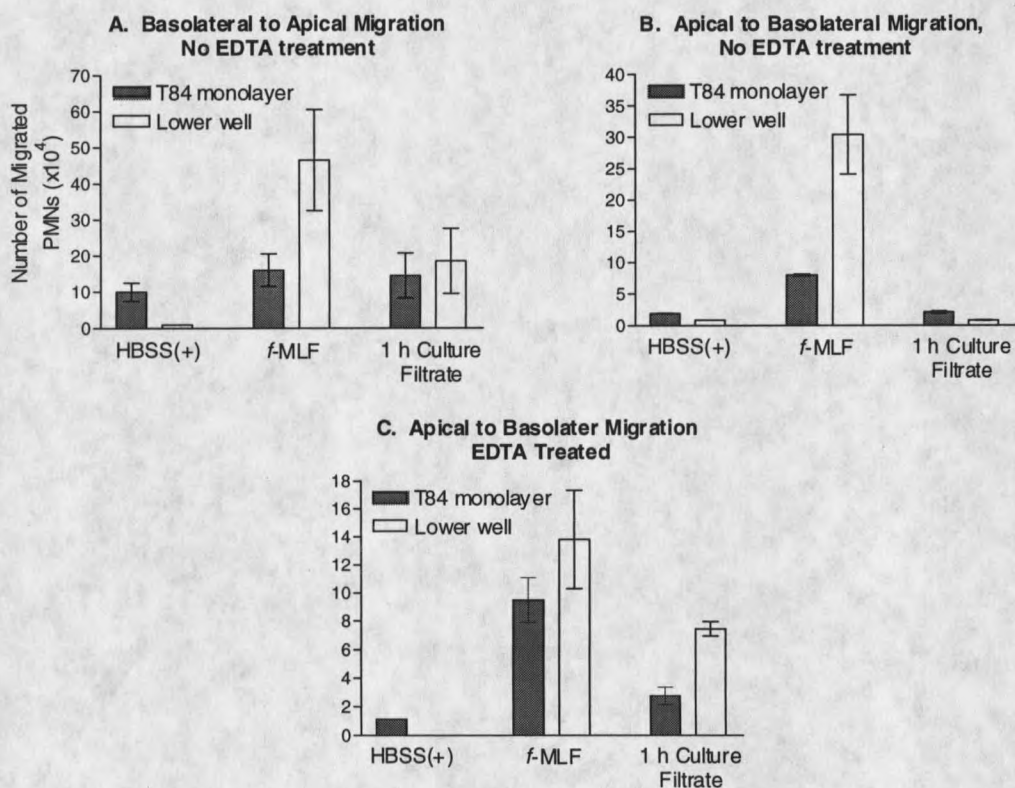


Figure 5. Transmigration Through a Monolayer of T84 cells Toward 1-h *C. albicans* Culture Filtrate. (A) Transmigration in the physiological direction, basolateral-to-apical. (B) Transmigration in the non-physiological direction, apical-to-basolateral, without the removal of extracellular Ca^{2+} . (C) Transmigration of cells in the non-physiological direction with prior treatment of the T84 cells with EDTA to remove extracellular Ca^{2+} that results in breaking epithelial tight junctions (C). For figures A and C, 1×10^6 PMNs per monolayer and f-MLF at 100 nM was used. For figure B, 2×10^6 PMNs per monolayer and f-MLF at 1 μM was used. PMN transmigration was assessed by quantification of the total number of PMNs found in the T84 cell monolayer and lower reservoir by the myeloperoxidase assay. The data are expressed as the mean \pm SD from a single representative experiment of three separate experiments. Determination of the number of PMNs contained within the monolayer was only performed for the data shown. For each experiment, all samples were run in triplicate.

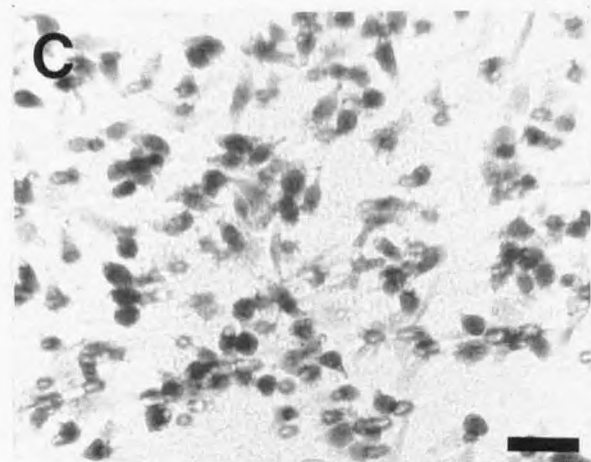
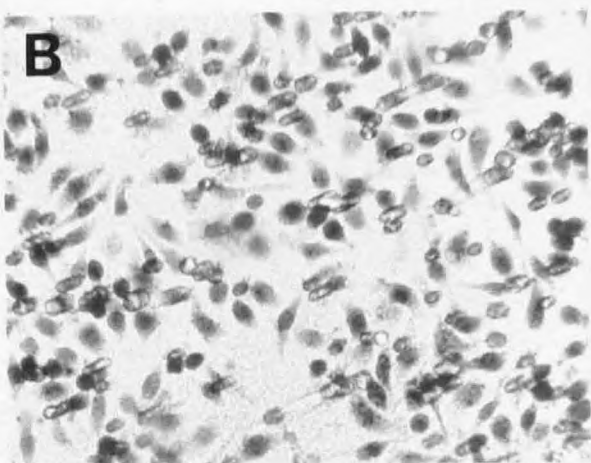
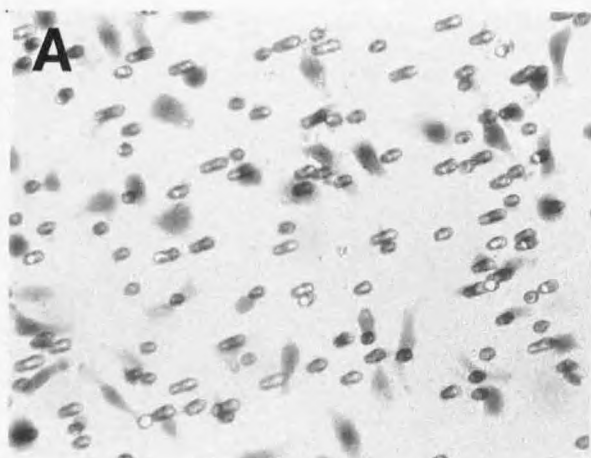
To determine if the polarity of the monolayer affected the PMN transmigration, we also examined the chemotaxis in the opposite direction (apical to basolateral). As expected, based on previous results by Parkos *et al.* (311), the 1-h culture filtrate was unable to induce transmigration in the non-physiological direction, from the apical side to the basolateral side (Fig. 5B). A total of 2×10^6 PMNs per monolayer and *f*-MLF at a concentration of $1 \mu\text{M}$ was used for assessing transmigration in the apical to basolateral direction because the efficiency of PMN migration in this direction is 5 to 20-fold lower than the basolateral to apical direction (310). However, transmigration in the apical to basolateral direction towards the culture filtrate could be induced after transient disruption of epithelial tight junctions by Ca^{2+} chelation (309) (Fig. 5C). Thus, NSCF can drive polarized transmigration in a physiologically relevant direction, but failed to induce migration in the reverse direction unless barrier function was disrupted.

Finally, to confirm that the culture filtrate itself did not damage the epithelial monolayer or alter the tight junction permeability, we examined the monolayer resistance. After a 5-h incubation with culture filtrate in the apical compartment and HBSS(+) in the basolateral compartment, or vice versa, no change in resistance was observed (data not shown). Activation of superoxide production by PMNs may also effect the epithelial monolayer while at the same time alter the myeloperoxidase assay results. Therefore, we determined whether the *C. albicans* culture filtrate stimulated superoxide production from PMNs using a cytochrome c microplate assay (65,317). The culture filtrate did not stimulate the production of superoxide from PMNs, suggesting that the culture filtrate does not activate superoxide production (data not shown). Superoxide production was only observed after the addition of phorbol 12-myristate 13-acetate and did not occur in the presence of superoxide dismutase (data not shown).

Chemotaxis of the Murine Macrophage-like Cell Line, J774

In addition to PMNs, macrophages are also important in host defense against disseminated candidiasis (325). A J774 cell line was chosen as a model of murine macrophages and tested for chemotactic activity toward the NSCF. J774 cells displayed chemotaxis toward a *C. albicans* 1-h culture filtrate (Fig. 6 and 7). An activity trend over time similar to that for PMNs was noted (Fig. 7), except that the highest chemotactic activity occurred at 0.5 h instead of at 1 h. The filtrate from a 1-kDa ultrafiltration induced chemotaxis of J774 cells, suggesting that the size of the NSCF for J774 cells was similar to that for PMNs (data not shown, Table 2). We were unable to induce chemotaxis of J774 cells toward *f*-MLF (10^{-5} to 10^{-10} M) (data not shown), which corresponded with evidence that murine macrophages do not express a high affinity FPR (137). Furthermore, we examined if mannoproteins released from *C. albicans* were responsible for the migration of J774 cells, since J774 cells have previously been shown to express the mannose receptor (38). Chemotaxis experiments were performed using a wide concentration range of 2- β -mercaptoethanol (2-ME) *C. albicans* cell wall extract (10^{-4} – 10^3 μ g/ml) diluted in HBSS(+). This extract contains the *C. albicans* cell wall phosphomannan complex, primarily mannan with about 3.5% protein, that has been identified as responsible for attachment of *C. albicans* to the splenic and lymph node macrophages in mice (181). No significant chemotaxis toward 2-ME extract above the negative control was observed for J774 cells. Stability of the J774 NSCF was also noted to differ from the PMN NSCF (Table 2). Neither heating at 56°C for 30 min nor boiling for 10 min decreased the chemotactic activity. These results suggest that the NSCF stimulating J774 cell chemotaxis produced by *C. albicans* yeast cells is different than the *C. albicans* NSCF for PMNs and interacts with an unknown receptor on J774 cells.

Figure 6. J774 Cells Migrate Toward *C. albicans* 1-h Culture Filtrate. Migration of J774 cells through 8.0 μm semi-porous supports for 4 h, at 37°C, toward (A) HBSS(+), (B) zymosan A activated human serum, and (C) 1 h culture filtrate. J774 cells adhered to the underside of the support were stained with hematoxylin. Bar, 50 μm . (data not shown). In addition, pretreatment of J774 cells with the 2-ME extract did not decrease J774 chemotaxis toward the *C. albicans* 1 h culture filtrate.



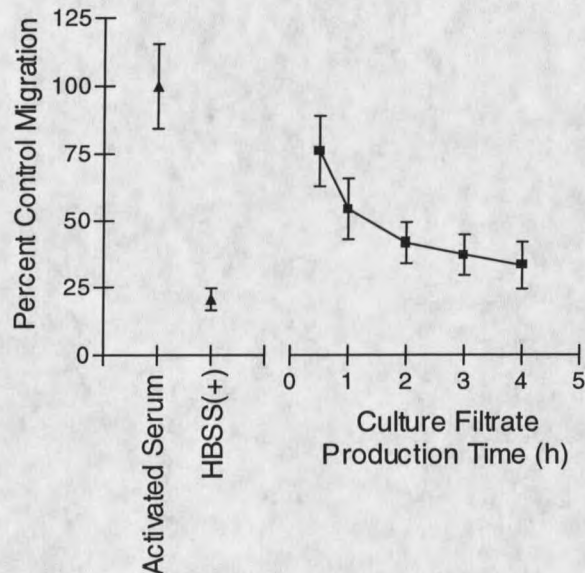


Figure 7. *C. albicans* Culture Filtrate Contains a Chemotactic Factor for the Murine Macrophage-like Cells, J774. Chemotaxis of J774 cells towards *C. albicans* 0.5, 1, 2, 3 and 4 h culture filtrates (■). Chemotaxis was assessed by quantification of the average cell area of hematoxylin stained J774 cells that adhered to the underside of the porous support. Activated serum was used as the positive control and HBSS(+) was used as the negative control. The average cell area of 10 randomly chosen 40x fields was determined per sample. The data are expressed as the mean \pm SEM from three different experiments. Data points were equalized by setting the migration of J774 cells toward zymosan A activated serum to 100.

Discussion

Previous studies have shown that *C. albicans* yeast cells produce a NSCF for PMNs (49,50,84,405). Our results confirmed and extended the scope of the previous findings. We showed that FPR-mediated chemotaxis accounted for approximately half of the PMN chemotaxis toward NSCF(s) in the *C. albicans* culture filtrate. Furthermore, we demonstrated that the NSCF(s) attracts PMNs across an intestinal epithelial cell monolayer *in vitro*. We also provide evidence for an additional chemotactic agent that stimulates chemotaxis of the murine macrophage-like cell line, J774, by a different receptor.

We found similarities and differences between the NSCF described here and those described by others. Consistent with other studies that implicated a role for a NSCF in cutaneous candidiasis (50), we found that the *C. albicans* NSCF is produced in the absence of glucose. NSCF chemotactic activity was found to peak at 1 h incubation at a concentration of 5×10^8 yeast cells/ml, whereas others have demonstrated that up to 12 h is necessary for production of chemotactic activity (84). Such discrepancies might be due to differences in concentration of yeast, the chemotactic assays, and the species from which PMNs were obtained. When a lower concentration of yeast was used, a longer incubation time was required for chemotactic activity to equal that of 5×10^8 yeast cells/ml. Others have noted a correlation between virulence and decreased stimulation of PMN chemotaxis by specific *C. albicans* strains (422). However, in results reported here, *S. cerevisiae* (a species rarely implicated in disease) and all *Candida* spp. tested produced a NSCF for human PMNs. In addition to these findings, others have shown that *Trichophyton mentagrophytes* and *Blastomyces dermatitidis* release low molecular weight chemotactic substances for PMNs (391,396). Therefore, a wide range of fungi can produce non-serum dependent PMN chemoattractants.

The decrease in chemotactic activity observed with a higher dose of *C. albicans* might be expected if an inhibitor of chemotaxis was produced after the initial production of the NSCF. The decrease in activity did not result from saturating amounts of chemoattractant, since NSCF activity could not be rescued by dilution of a 4 h culture filtrate (data not shown). It is also possible that denaturation or degradation of the NSCF occurs such that it no longer had chemotactic activity.

Our data implicated FPR as a receptor for a *C. albicans* NSCF. Preincubation of CHO-FPR and PMN with FPR antagonists significantly decreased chemotaxis toward the culture filtrate (Fig. 4). Standard ligands for FPR consist of bacterial and mitochondrial peptides that are synthesized with *N*-formylmethionine as the starting residue

(59,246,355,356,361). Some nonformylated peptides have also been shown to stimulate FPR-mediated chemotaxis but, with the exception of a few cases (135), the chemotactic activity of these peptides was significantly lower than that of their formylated counterparts (126,401). If the NSCF that interacts with FPR is a formylated peptide, the most obvious place of origin would be the mitochondrion. *C. albicans* mitochondrial proteins may be actively released or as a byproduct of yeast cell death during culture filtrate production. However, examination of cell death in cultures of 1 to 4 h does not support cell death as a source. Propidium iodide-stained cells analyzed by flow cytometry (249) showed that cell death did not increase with time but remained less than 1% throughout the production time, 1 to 4 h (data not shown). Because the antagonist only inhibited PMN chemotaxis toward the culture filtrate by approximately 50%, it is possible that other chemotactic factors containing in the culture filtrate and chemotactic receptors expressed by PMNs are involved in the observed chemotactic response. Other known chemotactic receptors expressed by human PMNs are: C5a receptor, C3a receptor, platelet activating factor receptor, C-X-C chemokine receptors (such as IL-8 receptor A and IL-8 receptor B), and C-C chemokine receptor 1. The ligands for the chemokine receptors are about 8-10 kD, suggesting that these receptors are unlikely candidates for binding NSCF unless they also bind small molecular weight factors. C5a receptor binds a 74 amino acid peptide but has also been shown to bind smaller peptides with lower affinity (209). However, CHO cells expressing C5a receptor showed no chemotaxis toward the *C. albicans* 1-h culture filtrate (data not shown), suggesting that NSCFs do not act as agonists for C5a receptor-mediated chemotaxis.

To investigate the relevance of our finding with respect to the GI tract, we used an in vitro T84 cell monolayer system to examine whether the *C. albicans* NSCF can attract PMNs through an epithelial monolayer. Due to the complexity of the GI tract, a reductionistic approach to unraveling the interactions of *C. albicans* at the GI epithelium

as a commensal and potential pathogen is necessary. As shown here, this *in vitro*-model system is useful for identification of putative *C. albicans* colonization factors and mechanisms of dissemination from the human GI tract. The *C. albicans* culture filtrate induced transmigration of PMNs in the physiological direction across a T84 epithelial cell monolayer (Fig. 5A), suggesting that the release of small molecular weight molecules by *C. albicans* helps to recruit PMNs into the gut. Since NSCFs induced chemotaxis of PMNs in the absence of the T84 cells, the factor(s) is not likely to be epithelial-derived. However, it is possible that the NSCF induced T84 cells to release other chemotactic agents, such as IL-8, which stimulate PMN transmigration. Because *f*-MLF has been shown to cross model intestinal epithelial monolayers by the paracellular pathway (397), it is highly probable that the NSCF contained in the culture filtrate is crossing the T84 monolayer by the paracellular pathway to stimulate PMNs transepithelial migration. The production of secreted aspartyl proteinases by *C. albicans*, has been suggested to facilitate hematogenous dissemination from the gut by digesting the mucin layer (79) and has also been shown to be chemotactic as well as chemokinetic for human PMNs (405). Thus, secreted aspartyl proteinases and NSCFs described in this study may act together to stimulate PMN infiltration.

We examined also whether *C. albicans* culture filtrate stimulated the migration of the macrophage cell line, J774. To our knowledge this is the first report of a non-serum dependent chemotactic factor produced by *C. albicans* for macrophages. Unlike the NSCF for PMNs, the production of the chemotactic factor for J774 cells peaked sooner and the activity remained stable when the 1-h culture filtrate was boiled for 10 min. In addition, despite using a wide concentration range of *f*-MLF (10^{-5} M to 10^{-10} M), we were unable to stimulate J774 cell migration, suggesting that they lack FPR expression and the NSCF contains a factor which is a ligand for a separate chemotactic receptor. These results support existing data that murine macrophages may lack expression of a high

affinity FPR (137,416).

We provide evidence in this study that *C. albicans*, along with other yeast species, produces a NSCF for human PMNs which may have immunoregulatory activity at sites where there is decreased complement activity. The ability of the culture filtrate to induce transmigration of PMNs across the T84 monolayer from the basolateral to the apical side suggests that the NSCF influences the host-pathogen interactions in the GI tract by stimulating an infiltration of leukocytes.

CHAPTER 3

IN VITRO ADHESION OF *CANDIDA ALBICANS* TO HUMAN
GASTROINTESTINAL MONOLAYERSIntroduction

The gastrointestinal (GI) tract is a major portal of entry for the hematogenous spread of *Candida albicans* in the immunocompromised host (73,188). The *C. albicans* strain residing on the mucosa is usually the primary agent of disseminated candidiasis (327,430). Therefore, the ability of *C. albicans* to adhere to and persistently colonize the mucosal surface of the human GI tract is considered a putative virulence trait (85). Although little is known about the maintenance of *C. albicans* as a commensal in the GI tract of healthy individuals, overgrowth on the mucosal surface and/or dissemination from the GI tract is apparently uncommon without immunosuppression of the host (76,188,297,298). Both specific and non-specific interactions are thought to play a role in *C. albicans* adhesion to the GI mucosa (76). Specific interactions of host-fungal cell-surface molecules are suggested by the preferential association of *C. albicans* with stratified epithelium of the cardium-atrium fold in the murine stomach (76), and the adherence of *C. albicans* yeast to an individual villi in the intestine without attachment to the surrounding villi (78).

In this study, we used monolayers of human colonic intestinal epithelial cell lines, T84 and Caco2, to compare their interaction with *C. albicans* and other yeast cells. These cell lines form confluent monolayers with high resistance to passive ion flow that correlates with tight junction formation and normal polarization. They display phenotypic similarities to intestinal crypt epithelial cells and are functionally similar to native crypt epithelial cells (88,163,239).

Materials and Methods

Mammalian Tissue Culture

Maintenance of T84 cells in culture was the same as described in Chapter 2. Caco2 cells, a kind gift from Dr. Susan Weimer-Mackin, Harvard Medical School, Boston, Massachusetts, were grown in low glucose (1,000 mg/L) Dulbecco's modified Eagles' medium (DMEM) (Sigma Chemical Co., St. Louis, MO) supplemented with 1.2 mg/ml NaHCO₃, 50 U/ml penicillin, 0.05mg/ml streptomycin, 3.58 mg/ml HEPES and 5% fetal bovine serum (FBS).

Yeast

All yeast strains were from the stock collection at Montana State University-Bozeman and included *C. albicans* strains 3153A, CA-1, A9, 105, and ATCC64550. Other yeast species in this study included *C. lusitaniae* (ATCC 64125), *C. parapsilosis* (ATCC 90018), *C. tropicalis* (ATCC 750), *C. glabrata* (ATCC 90030), and *S. cerevisiae* (2180WT). Identification of all species was confirmed with API 20C yeast identification strips (Analytab Products, Plainview, N.Y.). *C. albicans* strain 3153A was used in all experiments except where otherwise noted. All isolates were cultured from glycerol stock cultures held at -70°C and plated onto Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) for 48 h at 37°C.

Yeast were grown in 50 mM glucose-yeast nitrogen broth containing amino acids (glu-YNB (+)) (Difco Laboratories, Detroit, Mich.) at 22-23°C (RT), with shaking to stationary phase, then subcultured to fresh glu-YNB (+) and incubated to stationary phase. Yeast were subcultured to fresh glu-YNB (+) and grown at 30°C with shaking to stationary phase. Yeast were washed three times with RT saline and sonicated for 4 to 6 sec (Fischer Scientific water bath sonicator FS30, Fischer Scientific Co.) and checked microscopically to ensure that >95% were individual cells.

Radiolabeling Yeast

Blastoconidia were metabolically labeled with L-[³⁵S]-methionine (ICN, Irvine, CA) using a modification of the procedure by Rotrosen and colleagues (342). Yeast were suspended to 4.5×10^7 yeast/ml in 4.5 ml glu-YNB without amino acids containing 52.5 μ Ci of L-[³⁵S]-methionine (ICN, Irvine, Calif.). The yeast suspension was incubated for 2 h at 30°C, and aerated by shaking at 250 rpm (Controlled Environment Incubator Shaker model M52 New Brunswick Scientific Co., Inc. Edison, N.J.). Yeast were washed in saline seven times to remove the unincorporated radioactivity, sonicated, and suspended in Hank's balanced salts solution with calcium and magnesium [HBSS(+)] at pH 7.4, 22-23°C, at the desired cell concentration. Alternate growth and radiolabeling conditions included YNB containing 50 mM sucrose, 50 mM or 500 mM galactose, or 500 mM glucose, and 37°C.

Adhesion Assay

For adhesion assays, T84 and Caco2 monolayers were grown in RIA 96-well plates, medium-binding (Corning Costar, Inc., Cambridge, Ma.), precoated with rat tail collagen (88), and were used between 7 and 14 days in culture. Monolayers were washed three times with warm (37°C) HBSS(+), 100 μ l of the desired concentration of yeast was added to each well, and incubated at 37°C for 45 to 180 min without shaking. After the incubation, monolayers were washed four times with HBSS(+), and the washings containing non-adherent yeast were pooled into scintillation vials (Kimball Glass Co., Vineland, N.J.). RIA wells were separated and each was placed directly into scintillation vials. Scintisafe 30% (Fisher Chemical Co.), 5 ml, was added to each vial. β -emissions were counted for 2 min in a Scintillation Counter (Beckman, Fullerton, Calif.). The counting efficiency for samples containing 10 μ l or 400 μ l of HBSS(+) did not differ. All samples were run in triplicate. Sample cpm values were compared to standard curves created with each yeast species to convert cpm values into yeast concentration. The

percent adherent yeast per sample was then determined.

⁵¹Chromium Release Assay

Cellular damage of epithelial monolayers by *C. albicans* and *S. cerevisiae* was determined using a ⁵¹Cr release assay (119). Epithelial monolayers were grown in 12-well tissue culture plates for 14 days. Monolayers were incubated overnight at 37°C, plus 5% CO₂, in 500 µl tissue culture medium containing 6 µCi/ml Na₂⁵¹CrO₄ (ICN, Co., Costa Mesa, Calif.). The cell monolayers were washed three times with warm (37°C) HBSS(+), and 1 ml tissue culture medium containing yeast or medium alone was added to each monolayer and incubated as above for 3 or 6 h. Culture medium, 500 µl, was removed from each well and placed into scintillation vials. NaOH (6 N, 500 µl) was added to each monolayer, incubated for 15 min at 22-23°C without shaking, and the contents of each well was placed into scintillation vials. Each well was washed 2x, 500 µl each, with Count Off (Fisher Chemical Co.) and pooled with the appropriate vial. Scintisafe 30%, 2.5 ml, was added to each scintillation vial and cpm determined. The counting efficiency for samples with and without NaOH was 9.7 to 11% and 25 to 27%, respectively. All calculations of specific ⁵¹Cr release were carried out after converting cpm values to disintegrations per minute (127). Specific ⁵¹Cr release for each sample was determined as follows: Percent of total ⁵¹Cr per well released into the medium = $2 \times \frac{\text{released } ^{51}\text{Cr}}{\text{residual } ^{51}\text{Cr} + \text{released } ^{51}\text{Cr}}$, specific ⁵¹Cr release = $\frac{\text{percent experimental release} - \text{percent spontaneous release}}{1 - \text{percent spontaneous release}}$. The percent spontaneous release was the percent of total ⁵¹Cr released in wells containing monolayers without yeast that served as the negative control.

Results

Yeast adhesion to GI epithelial monolayers, T84 and Caco2, was monitored using L-[³⁵S]-methionine metabolically labeled yeast. Figure 8 shows a typical standard curve of radioactively-labeled *C. albicans* yeast. As reported by others (254), this labeling procedure was very sensitive. We were able to detect as low as 100 yeast per well. Yeast numbers were also determined by counting colony forming units from aliquots of the serial dilutions used for generating the standard curve. CFUs counts correlated with the yeast concentrations that had been determined by using a hemacytometer (data not shown).

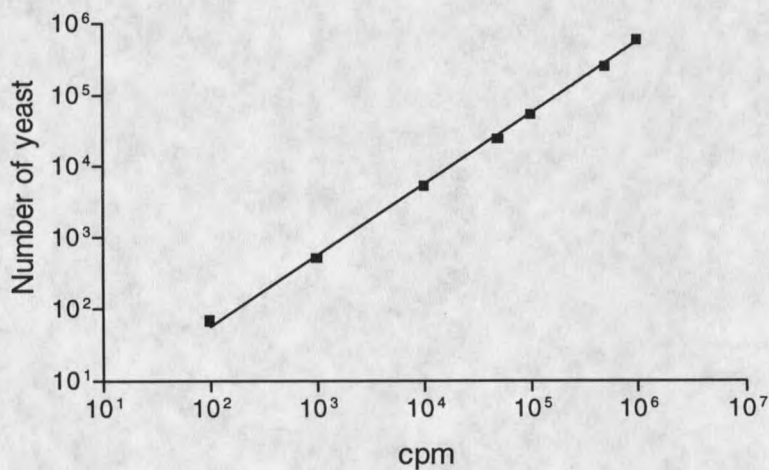


Figure 8. Typical Standard Curve of L-[³⁵S]-labeled *C. albicans* Yeast. Yeast were metabolically labeled with L-[³⁵S]-methionine as described in material and methods.

We first analyzed the adhesion of *C. albicans* and *S. cerevisiae* to T84 monolayers over a range of 10³ to 10⁵ yeast per monolayer (Fig. 9). *S. cerevisiae* was used because it is considered to be non-adherent to mammalian tissues *in vitro* (132). As can be seen, adhesion of *C. albicans* and *S. cerevisiae* yeast did not differ significantly when concentrations of 10³ and 10⁴ yeast per monolayer were used. This probably reflects non-specific adherence to the T84 monolayers. At 10⁵ yeast per monolayer,

adherence of *C. albicans* was significantly greater than *S. cerevisiae* (Student *t* test, $P = 0.0482$). Additionally, as the yeast inoculum size increased, the percent of *S. cerevisiae* yeast adhered to the T84 monolayer remained constant, whereas the percent of adherent *C. albicans* yeast increased (Fig. 9B). Based on these results, a 10^5 yeast per monolayer inoculum size was used for subsequent adhesion assays.

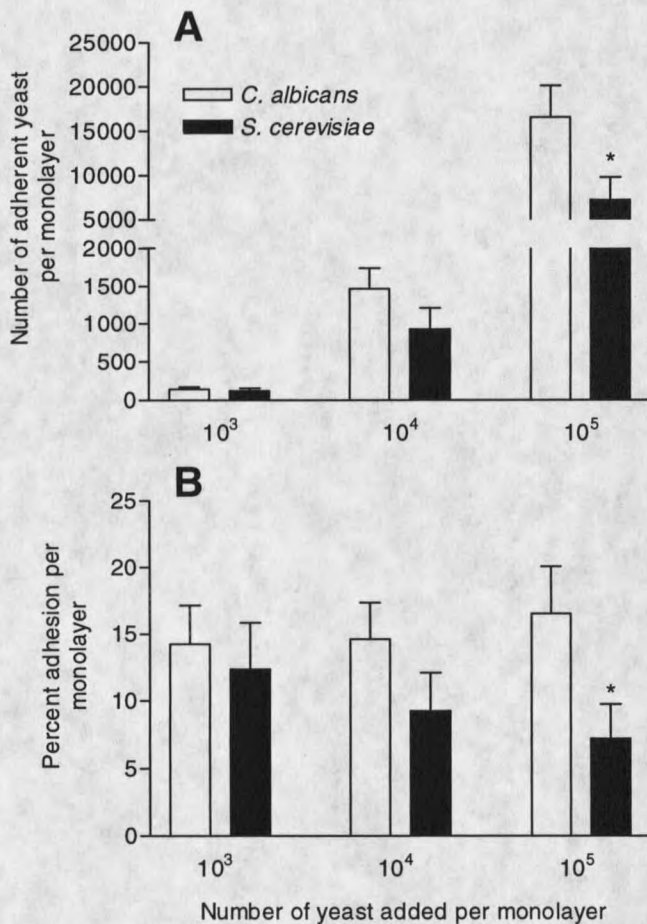


Figure 9. Effect of Yeast Dosage on Adhesion to T84 Monolayers. A range of inoculum sizes of L-[35 S]-labeled *C. albicans* and *S. cerevisiae* yeast were allowed to interact with T84 monolayers for 45 min at 37°C. Results are expressed as the total number of yeast adhered per monolayer (A), and the percent of adherent yeast per monolayer (B) from three separate experiments with all samples run in triplicate. Error bars indicate the standard error of the mean. *, Student's *t*-test $P = 0.0482$.

We next examined the adhesion of a variety of *Candida* species and *S. cerevisiae* to T84 and Caco2 monolayers (Fig. 10). *C. albicans* displayed the greatest adherence to the T84 monolayers (Fig. 10A). The order of adherence to T84 monolayers correlated well with the reported prevalence of *Candida* spp. in the human GI tract (297). To make sure that the adherence of *C. albicans* was not strain-specific, the adherence of five *C. albicans* strains to T84 monolayers was evaluated. The adherence of all *C. albicans* strains tested did not appear to differ (data not shown). The order of adherence to Caco2 monolayers was similar as that to T84 monolayers, except that *C. glabrata* displayed significantly greater adherence than the other yeast tested (Fig. 10B). *C. tropicalis* adhered poorly to both cell lines (Fig. 10). In addition, *C. albicans* adhesion to T84 and Caco2 monolayers was found to increase with time (Fig. 11). Radiolabeled yeast were

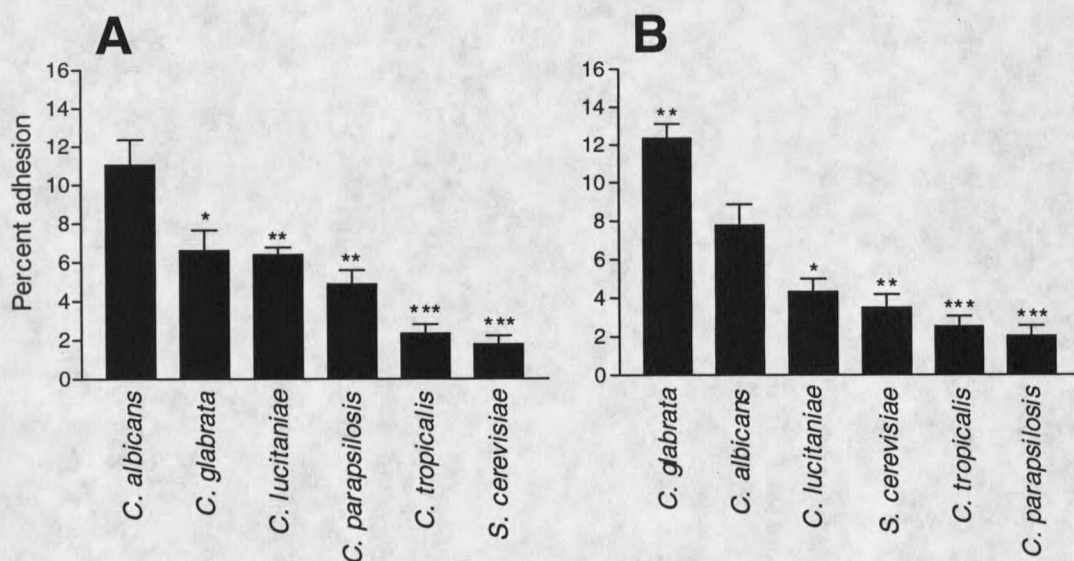


Figure 10. Adhesion of a Variety of *Candida* spp. and *S. cerevisiae* to GI Epithelial Monolayers. Adhesion was assessed to (A) T84 and (B) Caco2 monolayers using an inoculum size of 10^5 L- $[^{35}\text{S}]$ -labeled yeast per monolayer. Yeast were allowed to adhere to monolayers for 45 min. Figure A is two separate experiments with samples run in triplicate. Figure B is from four separate experiments, except data for *C. tropicalis* is from three separate experiments. Samples were run in triplicate. * Student's *t* test, *P*-values ranged from 0.026 to 0.0145. ** Student's *t* test, *P*-values ranged from 0.0094-0.0024. *** Student's *t* test, *P*-values ranged from 0.0003 to <0.0001. All *P*-values were calculated by comparing *C. albicans* to other yeast tested.

allowed to adhere to monolayers up to 180 min. Adhesion to T84 monolayers started to level off at approximately 135 min., whereas an increase in adhesion to Caco2 monolayers was still evident at 180 min, suggesting the monolayers may be displaying different surface properties or antigen phenotypes.

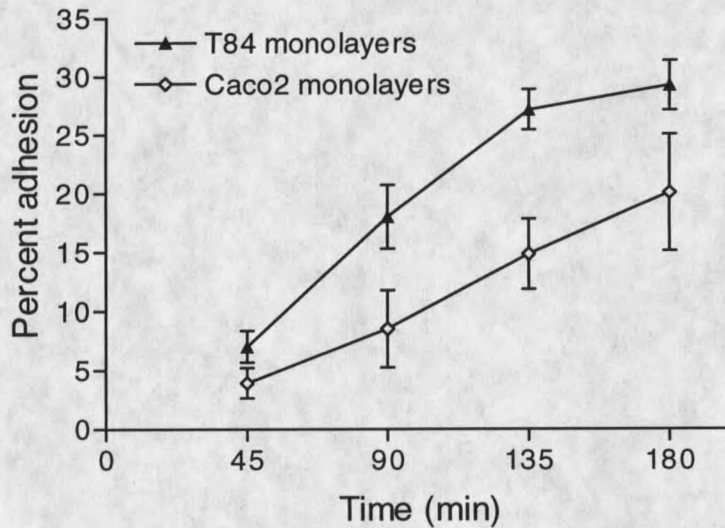


Figure 11. Percent Adhesion of *C. albicans* to GI Epithelial Monolayers is Time Dependent. L-[³⁵S]-labeled *C. albicans* yeast, 10⁵ yeast per sample, were allowed to adhere to either T84 or Caco2 monolayers for 45 to 180 min. Figure is a representative experiment from two experiments. Samples were run in triplicate Error bars represent standard deviation.

The percent adherence of *C. albicans* (Fig. 9-11) was found to decrease with increasing passage number of T84 monolayers. We used passage numbers 61 through 69 for the experiments. This result may possibly have been caused by a loss of expression of adhesion molecules on T84 monolayers, possibly explaining the decreased adhesion observed in the experiments performed with later passages (experiments shown in figures 2 through 4).

The type and concentration of carbon source and temperature used to grow *C. albicans* yeast has been suggested to influence the adherence characteristics of *C. albicans* (108). We observed no difference in adhesion to T84 or Caco2 monolayers

yeast grown in YNB containing 50 mM and 500 mM glucose and galactose, and 50 mM sucrose (data not shown). Additionally, yeast grown and radiolabeled at 37°C versus 30°C did not display different adhesion characteristics (data not shown).

It has been previously demonstrated that *C. albicans*' cell wall components, such as chitin (224) or mannoprotein (221,350), mediate adhesion of *C. albicans* to epithelial cells. Therefore, we attempted to inhibit the adhesion of *C. albicans* to T84 monolayers with *C. albicans* cell wall 2- β -mercapto ethanol (2-ME) extract or a chitin soluble extract (CSE). The 2-ME extract contains *C. albicans* cell wall phosphomannoprotein complex (181). 2-ME extract was kindly provided by Dr. Yongmoon Han at Montana State University-Bozeman. We isolated chitin soluble extract (CSE) from crab shell chitin (Sigma Chemical Co., St. Louis, Mo.) using the method described by Segal and colleagues (224). CSE prepared from crab shell had been shown to display similar inhibitory activity as *C. albicans* cell wall CSE (224). Pretreating either yeast or monolayers with 2-ME extract (10 - 200 μ g/ml in HBSS(+)), or CSE (approximately 10 mg/ml in HBSS(+)) did not effect the adhesion of yeast compared to non-treated controls (data not shown). This suggested that adhesion was not mediated by either of these cell wall components.

Damage to the epithelial monolayers by *C. albicans* and *S. cerevisiae* was evaluated using a ^{51}Cr release assay. *C. albicans* did not damage T84 or Caco2 monolayers to a greater extent than *S. cerevisiae* (Fig. 12) even though *S. cerevisiae* adhered poorly to both cell lines compared to *C. albicans* and is considered to be non-pathogenic. However, this observation appears to be consistent with transmission electron microscopy studies in which yeast were observed inside mouse GI epithelial cells but the cytoplasmic contents of the epithelial cells did not appear to be disturbed (78). This morphological finding suggested that the yeast were able to pass through the epithelial cells by a non-destructive mechanism.

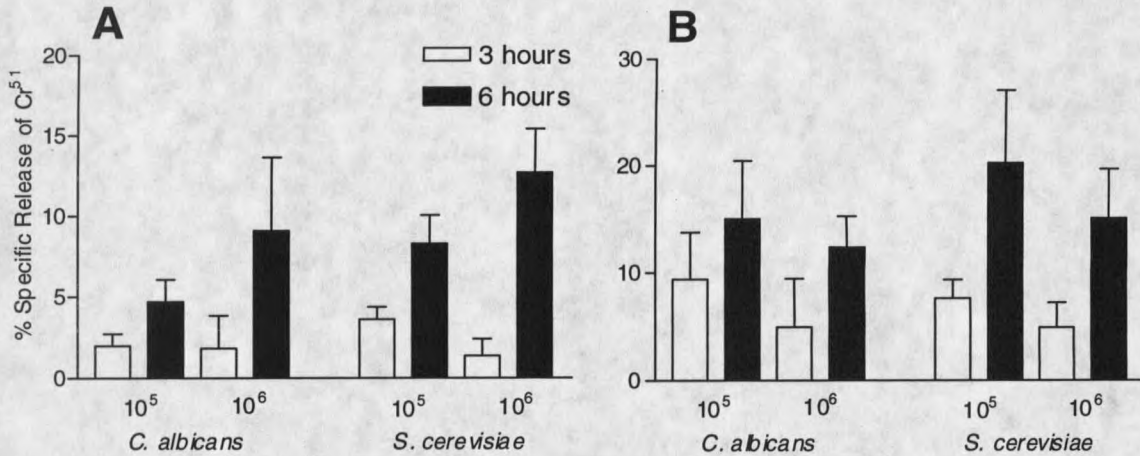


Figure 12. *C. albicans* Does Not Damage GI Epithelial Monolayers Greater than *S. cerevisiae*. T84 (A) or Caco2 (B) monolayers grown in 12-well plates were loaded with ^{51}Cr overnight. Two different concentrations of yeast, 10^5 or 10^6 yeast per monolayer, were allowed to interact with the monolayers for 3 and 6 h. The amount of yeast-induced cell injury was measured as the specific release of ^{51}Cr . These results are an average from two experiments. 10^5 or 10^6 yeast per monolayer. The yeast were allowed to interact with the monolayers for 3 and 6 h. Error bars represent standard deviation.

Discussion

In our study, we demonstrated that intestinal epithelial monolayers are a useful *in vitro* model to investigate host-commensal/pathogen interactions. We found that the degree of adhesion of five *Candida* species to GI epithelial monolayers, T84 and Caco2, (Fig. 10) correlated with the reported prevalence of *Candida* spp. in the human GI tract (297). Penetration through the GI mucosa by the endogenous *Candida* sp. is thought to be the most frequent mechanism leading to systemic dissemination in the immunocompromised patient (73,251,327,430). The most recent epidemiological analysis of *Candida* spp. nosocomial blood stream isolates (BSIs) (316) show that *C. albicans* accounted for 52% of total isolates followed by *C. glabrata* (18%), *C. parapsilosis* (15%), *C. tropicalis* (11%), and *C. krusei* (2%) (316). Therefore, data from our study together with epidemiological data, suggest that the ability to adhere to and

colonize the GI tract may play an important role in disseminated candidiasis in immunocompromised individuals.

Other researchers have compared the adhesion characteristics of various *Candida* spp. to different types of epithelial cells. The adhesion hierarchy to HeLa monolayers, a cervical epithelial carcinoma cell line, was as follows: *C. albicans* > *C. tropicalis* > *C. lusitaniae* \geq *C. parapsilosis* \geq *C. glabrata* = *C. krusei* = *S. cerevisiae* (30). This hierarchy correlated with the recognition of an integrin-like molecule on the yeast cells by the monoclonal antibody OKM1. This antibody recognizes human CD11b, the α subunit of β_2 -integrin CD11b/CD18 (30). As can be seen from our results (Fig. 10), *C. glabrata* and *C. tropicalis* displayed different adhesion characteristics to the GI epithelial monolayers, compared to HeLa cell monolayers. This difference suggests that *Candida* surface-expressed integrin-like proteins, that cross-react with OKM1, did not play an important role in adhesion of *Candida* spp. to the GI epithelial cell monolayers. However, we have not specifically evaluated the a role of yeast integrin-like proteins in adherence to these monolayers.

Results from our study also differed from yeast adhesion studies using exfoliated buccal and vaginal epithelial cells (199). Of the *Candida* spp. tested, *C. albicans* was found to be the most adherent to buccal and vaginal epithelial cells. Yet, unlike our study, *C. tropicalis* also displayed a significant amount of adherence to both buccal and vaginal epithelial cells, while *C. parapsilosis* and *C. glabrata* were virtually non-adherent (199). These results may reflect differences in cell adhesion molecules expressed on the different epithelial cell types and/or *Candida* species.

Although the adhesion to T84 and Caco2 monolayers by the yeast species tested was similar, differences were observed. We were surprised that *C. glabrata* displayed significantly greater adherence to Caco2 monolayers compared to the other *Candida* spp. This was unlike T84 monolayers where *C. albicans* displayed the greatest adhesion

compared to the other *Candida* spp. These results may reflect differences in cell adhesion molecules expressed by both the yeast and the epithelial cells. Because of the correlation between colonization of the GI mucosa and hematogenous dissemination in the immunocompromised host, these differences should be studied further to investigate the mechanisms used by the different *Candida* spp. to colonize the GI mucosa in humans.

CHAPTER 4

POLYMORPHONUCLEAR LEUKOCYTES FROM HOLSTEIN CALVES WITH BOVINE LEUKOCYTE ADHESION DEFICIENCY ARE DEFECTIVE IN PHAGOCYTOSIS AND KILLING OF *CANDIDA ALBICANS*Introduction

β_2 -integrins are cell surface glycoproteins that are made up of a common 95-kDa β chain, β_2 (CD18), that forms a heterodimer with one of four α chains: CD11a, CD11b, CD11c or CD11d (257,439). The β_2 -integrins are expressed in varying degrees by all human leukocytes and lymphocytes. Polymorphonuclear leukocytes (PMNs) express CD11a/CD18, CD11b/CD18 and CD11c/CD18 (257). β_2 -integrins play a role in both cell-matrix and cell-cell adhesion functions. For PMNs, this includes transendothelial (402) and transepithelial migration (308), and phagocytosis (52). β_2 -integrins also play a critical role in activation and upregulation of leukocyte killing mechanisms, such as superoxide production (241).

Leukocyte adhesion deficiency-type 1 (LAD-1) is an autosomal recessive disorder due to a genetic defect in the gene ITGB2 that encodes CD18 (200,379). This results in either severely reduced or complete absence of expression of functional β_2 -integrins (200). LAD-1 granulocytes (83), monocytes (242) and lymphocytes (93,256) all display abnormalities in a wide range of adhesion-dependent functions. Defects in PMN function are predominantly noted in transendothelial migration (419), respiratory burst regulation (367), chemotaxis (213,285) and phagocytosis (149,367). Although lymphocyte functions are also severely impaired, such as transendothelial migration and T cell activation (185,353), the pathology observed in LAD patients is primarily due to defective leukocyte function.

A number of animal models exist for studying the role of β_2 -integrins in adhesion-related functions of immune cells. These include a CD-18 null mouse with no expression of β_2 -integrins (353,419), a CD18-deficient mouse that has 2-16% expression of normal β_2 -integrin levels (436), and bovine LAD (BLAD) (186) in which calves display $\leq 2\%$ β_2 -integrin expression of normal PMNs (285,367). We have evaluated the phagocytic and killing capability of BLAD PMNs with *C. albicans* yeast. A phagocytic assay was used that distinguished adherent yeast from those that had been fully engulfed. In addition, we evaluated the contribution of opsonizing factors to phagocytosis by normal, heterozygous and BLAD PMNs.

Materials and Methods

Yeast

C. albicans, strain CA-1, was from the stock collection at Montana State University – Bozeman. Identification was confirmed with API 20C yeast identification strips (Analytab Products, Plainview, NY). CA-1 was cultured from glycerol stock cultures held at -70°C and plated onto Sabouraud dextrose agar (SDA) (Difco Laboratories, Detroit, MI) for 48 h at 37°C .

Source of Bovine Blood

Blood was drawn from age-matched normal, heterozygous and homozygous BLAD Holstein calves. The blood was collected into 50 ml polypropylene tubes containing acid citrate dextrose at the National Animal Center in Ames, IA, and shipped at room temperature to Montana State University – Bozeman. PMNs were isolated and used for experiments within 24 h of collection. Bovine serum was separated from blood isolated from a normal Holstein calf housed at our facility. Serum was held in an ice-water slurry if it was to be used the same day as isolated, or stored at -20°C until use.

Isolation of Bovine PMNs

Bovine PMNs were isolated using a distilled water lysis method. Blood, 50 ml per tube, was centrifuged at 300 x g for 20 min at 22-23°C. The plasma and buffy coat were removed and discarded. The red blood cells were lysed by the addition of 10 mls ice cold distilled water for approximately 10 sec, and then the total volume was increased to 50 ml with cold Dulbecco's phosphate buffered saline (DPBS), pH 7.4. Cells were pelleted by centrifugation at 400 x g for 10 min at 4°C. The water lysis was repeated a total of 3 times. Isolated PMNs were suspended in DPBS and held on ice until use. The purity of PMNs was approximately 90% as determined by visual inspection.

Production of FITC-Labeled Yeast

A single yeast colony from a SDA plate was inoculated into glucose (2%), yeast extract (0.3%), peptone (1%) broth (GYEPB), incubated to stationary phase (24 h) at 37°C with aeration. Yeast were subcultured to fresh GYEPB and incubated to stationary phase again. The yeast cells were then harvested by centrifugation, washed three times with DPBS at pH 7.4 and fixed with 10% formaldehyde in DPBS at 4°C for 30 min to 1 h, with gentle inversion every 5 min. The formalin-fixed yeast were washed three times with DPBS at 4°C and stored at 4°C until use. Heat-killed yeast were produced by boiling yeast suspended in DPBS for 30 min with gentle inversion every 5 min. Yeast were then washed three times with DPBS at 4°C and stored at 4°C until use. Aliquots of formalin-fixed and heat-killed yeast were plated on a SDA plate and incubated for 48 h at 37°C to check for sterility. All yeast were labeled with FITC as described by Lyman and Walsh (236). Briefly, heat killed yeast at 10^7 yeast cells/ml were washed once in room temperature 0.2 M PBS at pH 8.0 and incubated in 250 μ M FITC in 0.2 M PBS at pH 8.0 for 30 min in the dark at 22-23°C. The yeast were then washed three times with 0.2 M PBS at pH 7.4 and stored at 4°C.

Yeast Phagocytosis Assay

PMNs, 5.4×10^6 /ml, were suspended in prewarmed (37°C) RPMI-1640 (Sigma Chemical Co., St. Louis, MO) directly before use. The PMN suspension was added to Thermanox cover slips (Nunc, Inc., Naperville, IL) inside the wells of a 6-well tissue culture plate, total volume of 500 μl per coverslip. The PMNs were allowed to adhere to the coverslips for 30 min to 1 h at 37°C without shaking. The RPMI-1640 was removed and 500 μl of FITC-labeled heat-killed *C. albicans* yeast, 2.7×10^6 yeast/ml, in either RPMI-1640 plus 10% normal bovine serum, complement-inactivated bovine serum, or *C. albicans* yeast-absorbed serum, pre-warmed to 37°C , was added to the wells. Serum was complement inactivated by treating it at 56°C for 30 min. *C. albicans* yeast-absorbed serum was produced by mixing 1 part packed formalin-killed yeast cells with 10 parts of normal bovine serum for 10 min on ice. Yeast were then pelleted and the supernatant removed and treated two more times with fresh formalin-killed yeast. PMNs were incubated with the yeast for 1 h at 37°C without agitation. To stop the interaction, cold paraformaldehyde, final concentration 1%, was added to each well and the samples were incubated 1 to 2 h at $22\text{-}23^\circ\text{C}$ or overnight at 4°C . The FITC of attached yeast, but not ingested, was quenched by the addition of trypan blue, 2 mg/ml in 0.02 M citrate buffer containing 0.15 M NaCl at pH 4.4 (236). Trypan blue does not penetrate the PMN plasma membrane. The number of ingested yeast was determined by visually counting the number of fluorescent yeast associated with at least 100 PMNs per sample with a Nikon epifluorescent microscope. For each experiment, samples were run in duplicate.

Yeast Killing Assay

To analyze the ability of PMNs to kill *C. albicans* yeast, 2.7×10^6 PMNs and 5.3×10^6 live yeast were combined in a total volume of 1 ml RPMI-1640 plus 10% normal bovine serum and incubated for 1 h at 37°C with rotation. The samples were then serially

diluted in DPBS and aliquots were plated onto SDA plates. After incubating for 48 h at 37°C, yeast CFUs were counted. All samples were run in triplicate.

Results

We compared the phagocytic capability of PMNs isolated from normal, heterozygous and homozygous BLAD Holstein calves using *C. albicans* yeast cells. Three different conditions were tested, phagocytosis in the presence of normal, yeast-absorbed, or complement-inactivated bovine serum. As can be seen in figure 13, with all conditions tested, BLAD PMNs were almost completely deficient in phagocytic ability. Figure 13 is a representative experiment of three separate experiments. Variation was observed in the measured phagocytic index from experiment to experiment. The phagocytic index of BLAD PMNs for the other two experiments ranged from 0 to 0.06 for all conditions tested. The phagocytic ability of heterozygous PMNs was

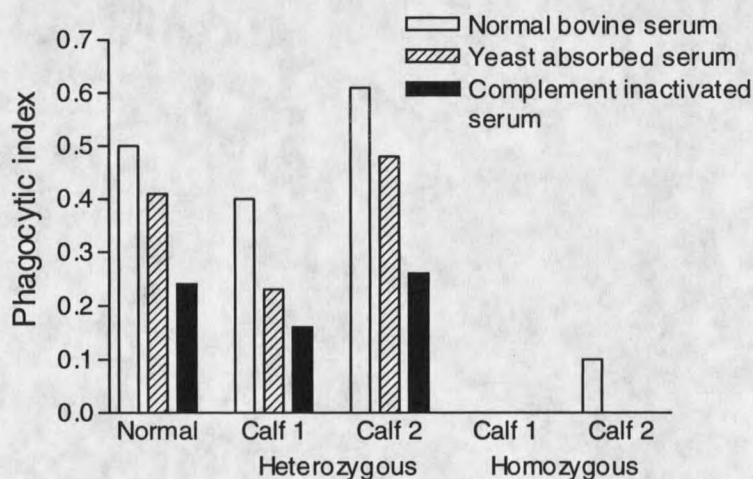


Figure 13. Phagocytosis of *C. albicans* Yeast by PMNs Isolated from Normal, Heterozygous, and Homozygous BLAD Calves. Bovine PMNs were incubated with FITC-labeled *C. albicans* suspended in normal bovine serum (open bars), yeast absorbed serum (striped bars), or complement inactivated serum (filled bars) and the number of phagocytosed yeast per PMNs was determined by fluorescence microscopy as described in materials and methods. Samples were run in duplicate. Figure is from a representative experiment. The experiment was repeated three times.

indistinguishable from normal PMNs in all three experiments, and serum complement inactivation or absorption with yeast always resulted in a decreased phagocytic index of normal and heterozygous PMNs compared to untreated serum. Although, a lower phagocytic index was not always observed for the absorbed serum compared to complement-inactivated serum.

As can be seen in figure 14, BLAD PMNs did maintain the ability to kill *C. albicans* yeast. Although, their killing efficiency was greatly decreased compared to both normal and heterozygous PMNs. Similar to the phagocytosis results, the killing efficiency of heterozygous PMNs was indistinguishable from normal PMNs.

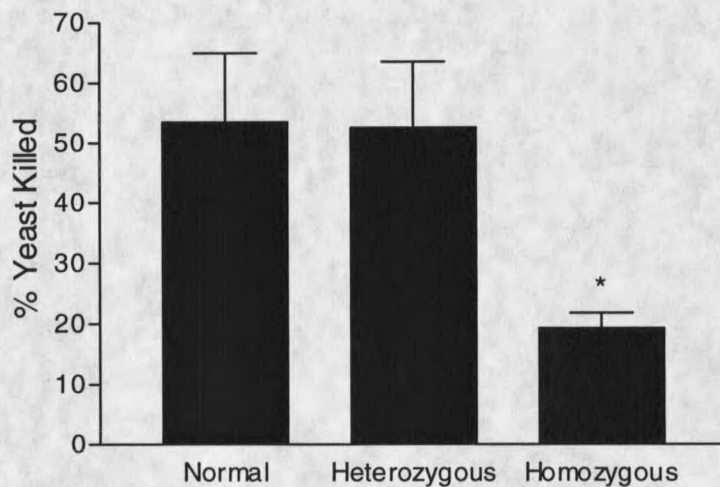


Figure 14. Killing of *C. albicans* Yeast by PMNs Isolated from Normal, Heterozygous, and Homozygous BLAD calves. PMNs, isolated from normal, heterozygous or homozygous BLAD calves, were combined with yeast in RPMI-1640 plus 10% normal bovine serum for 1 h at 37°C. An effector to target ratio of 1:2 was used. Yeast survival was determined by counting CFUs. Error bars indicate standard deviation. Samples were run in triplicate. * Student's *t* test, $P < 0.005$ for homozygous versus both normal and heterozygous PMNs.

Discussion

CD11b/CD18 is considered the principle leukocyte adhesion receptor for *C. albicans* (123). Our results support this conclusion. BLAD PMNs were severely deficient in phagocytosis of *C. albicans* yeast. In addition, adhesion of yeast to BLAD PMNs was absent (data not shown). These results are similar to those of other groups that evaluated the phagocytic ability of LAD PMNs with *S. cerevisiae* (286) and zymosan particles (9). We did not observe a difference in the phagocytic index of normal and heterozygous bovine PMNs, suggesting that heterozygous PMNs have normal β_2 -integrin expression. Phagocytosis of yeast by normal and heterozygous PMNs with *C. albicans* yeast-absorbed serum and complement-inactivated serum was decreased, but not absent. Therefore, neither antibodies nor complement components were necessary for phagocytosis of *C. albicans*, although they were required for maximum phagocytosis by bovine PMNs.

Cooperation of CD11b/CD18 with Fc γ receptors, Fc γ RIIa and Fc γ RIIIb, results in enhanced adhesive capabilities of PMNs (399). Although, cell effector functions mediated by Fc γ RI (CD64), the high affinity receptor for monomeric Fc γ (314,339), are not thought to be dependent upon CD11b/CD18, and may actually be enhanced in LAD patients (214,242). Compared to normal PMNs, investigators showed unstimulated LAD PMNs displayed enhanced expression of all three Fc γ receptors (Fc γ Rs), Fc γ RI (241), Fc γ RIIa (440) and Fc γ RIIIb (281,283,440). LAD PMNs, but not normal PMNs, displayed enhanced phagocytosis and chemiluminescence, representing superoxide production, in response to murine IgG2a-opsonized sheep RBCs (241). Both of these responses were inhibited by monomeric human IgG, suggesting they were Fc γ RI-mediated (241). In addition, human recombinant IFN- γ (hrIFN- γ) was used to treat one LAD patient to try to increase leukocyte Fc γ RI (CD64)-mediated effector functions (423). Although the patient died after 10 weeks of treatment, due to surgical

complication, the patient had not experienced any new infections or side-effects due to the hrIFN- γ since treatment was initiated (423). This suggested that hrIFN γ treatment may be a way to restore some phagocytic cell effector functions. We attempted to pre-treat BLAD PMNs with hrIFN- γ to try to enhance their phagocytic ability. Human rIFN- γ was used instead of bovine IFN- γ because bovine IFN- γ was not available. We did observe enhanced phagocytosis of *C. albicans* yeast by BLAD PMNs, suggesting it did not stimulate enhanced expression or function of Fc γ RI (data not shown). It is possible that the hrIFN- γ was incompatible with bovine PMNs.

Even though PMNs from BLAD calves were unable to engulf the yeast (Fig. 13), they still maintained approximately 30% of the capacity of normal or heterozygous PMNs to kill *C. albicans* yeast (Fig. 14). BLAD PMNs were observed to generate approximately twice the amount of superoxide anion than normal PMNs when stimulated with PMA (367). It is possible that even without phagocytosis, oxidative-dependent mechanisms were responsible for the killing observed in our assays. Even so, the killing activity of BLAD PMNs was severely decreased, suggesting that phagocytosis was necessary for efficient killing.

CHAPTER 5

CONCLUSIONS

1. Non-serum dependent chemotactic factors (NSCFs) produced by *Candida albicans* yeast stimulated chemotaxis of human PMNs through the formyl peptide receptor (FPR). This was based on the observation that NSCF-induced chemotaxis of PMNs and Chinese hamster ovary cells expressing human FPR was inhibited by FPR antagonists. However, because PMN chemotaxis was not totally inhibited, we cannot rule out the possibility that the culture filtrate contained other chemoattractants recognized by neutrophil chemoattractant receptors other than FPR.
2. *C. albicans* culture filtrates also stimulated chemotaxis of the murine macrophage cell line, J774, through an unknown receptor. To our knowledge, this was the first report of a non-serum dependent chemotactic factor produced by *C. albicans* for macrophages.
3. *C. albicans* culture filtrate containing NSCFs was found to stimulate PMN transepithelial migration in the physiological direction, basolateral to apical, but not the reverse direction unless the epithelial tight junctions were disrupted. This suggested that NSCFs produced by *C. albicans*, and other yeast species, may influence host-pathogen interactions at the GI mucosal surface by inducing phagocytic cell infiltration.
4. PMNs isolated from calves with BLAD, but not normal or heterozygous calves, displayed decreased phagocytosis and killing of *C. albicans* yeast. This supports studies claiming that the β_2 -integrin, CD11b/CD18, is the primary PMN adhesin for *C. albicans*, and is necessary for host defense against this opportunistic pathogen.
4. Human intestinal epithelial cell lines, T84 and Caco2, were found to be a useful *in vitro* model system to investigate the initial steps of colonization of the human GI tract. Adherence characteristics of five *Candida* spp. and *S. cerevisiae* suggested that different cell adhesion molecules expressed by the epithelial cells and/or *Candida* spp., mediated adherence to GI epithelium compared to exfoliated buccal and vaginal epithelial cells.

APPENDIX

FURTHER STUDIES ON THE INTERACTION OF *CANDIDA ALBICANS*
AND HUMAN GASTROINTESTINAL EPITHELIAL MONOLAYERSIntroduction

As previously stated, *Candida albicans* is commonly found as a commensal in the gastrointestinal (GI) tract of healthy individuals (72), and the GI tract is a major portal of entry for hematogenous spread of *C. albicans* in immunocompromised patients (73,188). Yet, the exact mechanism(s) used by *C. albicans* to pass through the GI mucosa in immunocompromised patients is still unknown. Both transcellular and paracellular transcytosis routes have been observed in infant mice inoculated intragastrically with *C. albicans* yeast (76). Certainly, damage to the integrity of the GI mucosa by cytotoxic drugs and irradiation aids in the translocation of *C. albicans* (46,68). Additionally, Alexander and colleagues observed a high frequency of *C. albicans* yeast translocation through the mucosal epithelium covering Peyer's patches of the small intestine (8), suggesting a role for M cells. These specialized epithelial cells (194) transport foreign antigen from the GI lumen directly to cells contained within intraepithelial pockets formed by the basolateral surface of M cells, including lymphoid cells, professional phagocytic cells, and antigen presenting cells (291). Antigen is transported by directed movement within membrane vesicles from the apical to basolateral surface and appears to be highly dependent upon the polarized nature of these cells (291). In addition, the apical surface of M cells differs dramatically from the surrounding enterocytes with a less-organized brush border (173), different surface antigen expression, including divergent glycosylation patterns (142), and recognition of IgA alone or complexed with antigen (291,429).

C. albicans transcytosis by M cells through the GI mucosa has not been investigated. This is partly due to the difficulty in studying M cells. They are relatively rare, constituting <0.1% of GI enterocytes (238), and specific cell markers for M cells are lacking. Therefore, identification of M cells has been based on their unique ultrastructural morphology compared to absorptive enterocytes. However, researchers have recently shown human M cells preferentially display the carbohydrate epitope, sialyl Lewis A antigen (sialyl Le^a) (142). In addition, an *in vitro* system was used to induce enterocytes, Caco2 cells, to switch to an M cell phenotype that was able to transport *Vibrio cholerae* across polarized monolayers (194). I attempted to use this *in vitro* switching system to investigate the role of M cells in transcytosis of *C. albicans* blastoconidia across GI epithelial monolayers *in vitro*. This report contains preliminary experimental data.

Materials and Methods

Yeast

C. albicans, strain 3153A, was from the stock collection at Montana State University-Bozeman. Identification was confirmed with an API 20C yeast identification strip (Analytab Products, Plainview, NY). The yeast inoculum for adhesion assays was prepared exactly as described in Chapter 3. Yeast were suspended in tissue culture medium at the desired concentration for transepithelial electrical resistance studies and in HBSS(+) at the desired concentration for adhesion assays.

Mammalian cell culture

T84 and Caco2 cell lines were maintained in culture exactly as described in Chapters 2 and 3. Inverted T84 monolayers were prepared as described in Chapter 2 and inverted Caco2 monolayers were prepared exactly the same way. The human B cell lymphoma cell line, Raji, was a kind gift from Dr. Jean Starkey, Montana State

University-Bozeman, Bozeman, MT. Raji cells were grown as cell suspensions in spinner flasks (Bellco Glass, Inc., Vineland, NJ), 37°C in the presence of 5% CO₂, in high glucose (4,500 mg/L) DMEM supplemented with 1.2 mg/ml NaHCO₃, 50 U/ml penicillin, 0.05mg/ml streptomycin, 3.58 mg/ml HEPES and 5% FBS. Hybridoma cloning factor (Origen Internation, Inc., Gaithersburg, MD), at the concentration suggested by the manufacturer, was added to Raji cell cultures for the first passage after thawing the cells from storage in liquid nitrogen.

Raji cells were added to T84 or Caco2 monolayers to induce M cell formation as described by Kernéis and colleagues (194). Briefly, T84 and Caco2 cells were grown as inverted monolayers until mature, generally 7 days. Cell monolayer maturity was determined by measuring the transepithelial electrical resistance with an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) (209), 175 and 334 $\Omega\text{-cm}^2$ for Caco-2 and T84 monolayers, respectively. A high transepithelial electrical resistance has been shown to correlate with tight junction (also known as occluding junction) formation and a mature enterocyte ultrastructural phenotype (240). Raji cells were pelleted, the supernatant discarded, and cells suspended in the medium that corresponded to the cell monolayer type that they were added to. Raji cells were added, at the indicated concentration, to the top well (basolateral side) of mature inverted T84 or Caco-2 monolayers in a total volume of 150 μl per well. Raji cells were allowed to interact with the epithelial monolayer for 12 h, 37°C plus 5% CO₂. After this, the upper chamber was washed three times with warm PBS at pH 7.4 with gentle repipetting to remove Raji cells that had not migrated into the epithelial monolayer. Fresh medium, 150 μl , was then added to the upper well. Culture medium in the upper and lower wells was replaced every 2 to 3 days. Co-cultures were incubated up to 7 days at 37°C before using them for adhesion assays and the transepithelial resistance of the monolayers was monitored daily.

Immunofluorescence Microscopy

For immunofluorescence microscopy with anti-sialyl Le^a antigen monoclonal IgG₁, KM231 (Kamiya Biomedical Co., Seattle, WA), T84 and Caco2 monolayers were grown on 8 well tissue culture slides (Corning Costar, Inc., Cambridge, MA or Nalge, Nunc International, Rochester, NY) coated with rat tail collagen (88). Monolayers were washed three times with warm HBSS(+) at pH 7.4, three times, and then fixed at 4°C for 30 to 60 min with periodate-lysine-paraformaldehyde fixative containing 2% paraformaldehyde (262). Monolayers were then washed once with HBSS(+) at 4°C, and two times at RT with Tris buffered saline, pH 7-8, containing 0.1% gelatin and 0.05% saponin (TBS). KM231 at 5 µg/ml in TBS was allowed to interact with the monolayers for 30 to 60 min at 22-23°C. After this, the monolayers were washed three times with TBS and incubated for 30 to 60 min at RT with a goat anti-mouse IgG FITC-conjugated secondary antibody (Sigma Chemical Co., St. Louis, MO). Monolayers were washed two times with TBS without saponin, five times with TBS without saponin or gelatin, and then three times with water. Coverslips were applied to the monolayers after the addition of glycerol-gelatin mounting medium containing 1,4-Diazobicyclo[2.2.2]Octane (Sigma Chemical Co., St. Louis, MO) and observed with a Nikon epifluorescence microscope.

For endogenous phosphatase detection on epithelial monolayers, the ELF-97 Endophosphatase Detection Kit (Molecular Probes, Eugene, OR) was used. The manufacturer's directions were followed. Samples were observed with a Nikon epifluorescence microscope equipped with 360 nm excitation and 520 nm emission filters.

Results

The Effect of pH on the Transepithelial Electrical Resistance of T84 Monolayers

The transepithelial resistance has been directly correlated with tight junction formation between the epithelial cells, and therefore the health of the mature monolayer (240). We monitored the electrical resistances of T84 monolayers after the addition of various concentrations of yeast. *C. albicans* yeast were allowed to interact with the apical surface of T84 monolayers for up to 34 hours (Fig. 15A). An initial rise in transepithelial electrical resistance was observed after the addition of yeast and appeared to be yeast dose dependent (Fig. 15A). The transepithelial electrical resistance of the monolayers with medium alone was identical to the starting resistance at the 6 hour time point. A small decrease in resistance was observed with control monolayers at approximately 9 hours, after which time a steady state was reached. We believe this may have been due to continual manipulation of the monolayers. The resistances of the monolayers with yeast showed a steady decrease that was most likely due to the destruction of the monolayer integrity by the yeast (Fig. 15A).

After the addition of the yeast to the T84 monolayers, we observed that the yeast began to acidify the tissue culture medium. Therefore, we decided to determine if this pH change was responsible for the initial increase in transepithelial resistance. DMEM was prepared without sodium bicarbonate and adjusted to 6.8 – 7.4 pH units, in increments of 0.2 units. The transepithelial resistance was monitored for 5 hours after the addition of the medium to the monolayers (Fig. 15B). An increase in transepithelial resistance was observed directly after the medium was added to the monolayers and was most likely due to handling of the monolayers, a phenomenon that we had previously observed. An inverse relationship between transepithelial resistance and pH, such that a small drop in pH stimulated an increase in the monolayer transepithelial electrical resistance was noted. Samples at pH 7.4 appeared to stabilize by 1 h and remain constant

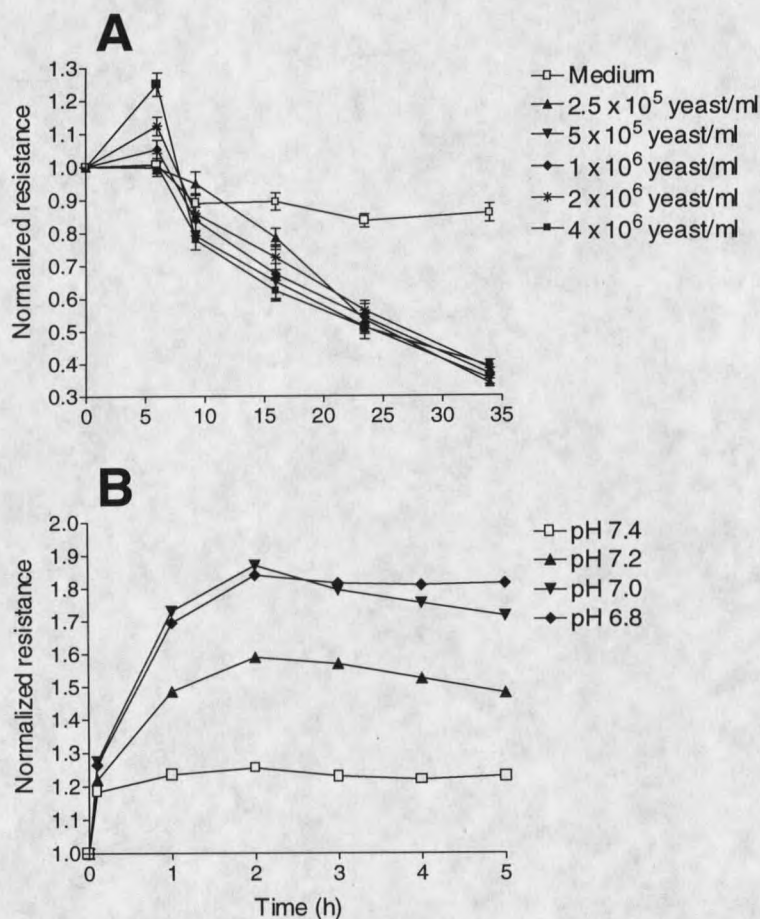


Figure 15. Effect of *C. albicans* Yeast and pH on T84 Monolayers Transepithelial Electrical Resistance. *C. albicans* yeast (A) or culture medium at various pH (B) were applied to mature T84 monolayers and the transepithelial resistances were monitored using an EVOM epithelial voltohmmeter. Transepithelial resistances were normalized to 1 for time = 0 hr; the time that the yeast or medium were applied to the monolayers. Figure A is a representative experiment from 3 separate experiments, samples were run in quadruplicate. Error bars indicate standard deviation. Figure B is a representative experiment from 2 separate experiments, samples were run in duplicate.

up to 5 h. The resistances of the other monolayers at pH 7.2 - 6.8 stabilized by the 2-h time point and remained elevated up to 5 h. Therefore, these results suggested that the increased resistance observed when *C. albicans* yeast were added to the monolayers was stimulated by the decreased pH of the tissue culture medium.

Co-culture of T84 or Caco2 Monolayers with Raji cells

Kernéis and colleagues demonstrated the ability of Peyer's patch cells or Raji cells to induce the conversion of Caco2 into M cells, as judged by ultrastructural changes and the ability of these cells to transport bacteria, *Vibrio cholerae*, across the monolayer in the apical to basolateral direction (194). Therefore, we thought it would be interesting to evaluate the interaction of *C. albicans* yeast with M cells using this *in vitro* system.

We originally attempted to isolate Peyer's patch cells from the mouse GI tract. Two separate protocols were used. The first consisted of dissociating Peyer's patches between glass slides and then placing cells into culture (194), and in the second, Peyer's patch cells were dissociated using collagenase 1A (Sigma Chemical Co., St. Louis, MO). Bacterial contamination and low cell number yields were associated with both methods. Therefore, induction of M cells by the addition of Raji cells was attempted.

Raji cells were added to the basolateral surface of inverted T84 and Caco2 monolayers. Kernéis and colleagues used a concentration of 10^6 Raji cells per inverted Caco2 monolayer grown on a 0.33 cm^2 filter with $5.0 \text{ }\mu\text{m}$ holes, to stimulate conversion of Caco2 cells to M cells (194). We noted a significant decrease in monolayer resistance was noted when this method was used (Fig. 16A), and the resistances failed to recover. Hematoxylin staining of Caco2 monolayers after the addition of Raji cells showed that a large portion of the Caco2 cells had detached from the porous support (data not shown), suggesting that the Raji cells had an adverse effect on the integrity of the Caco2 monolayers. Therefore, we were unable to further evaluate M cell production and interaction with *C. albicans* using Caco2 monolayers. An initial drop in resistance was also observed after Raji cells were added to T84 monolayers (Fig. 16B), but unlike the Caco2 monolayers the resistance did recover. As can be seen in figure 16B, a lower concentration of Raji cells did not appear to affect the resistance of T84 monolayers.

Yet, in other experiments a decrease in resistance has been observed with these Raji cell concentrations as well (data not shown). We observed a slight increase in adherence of *C. albicans* yeast to the apical side of T84 and Raji co-culture monolayers compared to control monolayers, approximately 2% from four separate experiments run in triplicate each. (data not shown). This increase was not significantly different compared to controls.

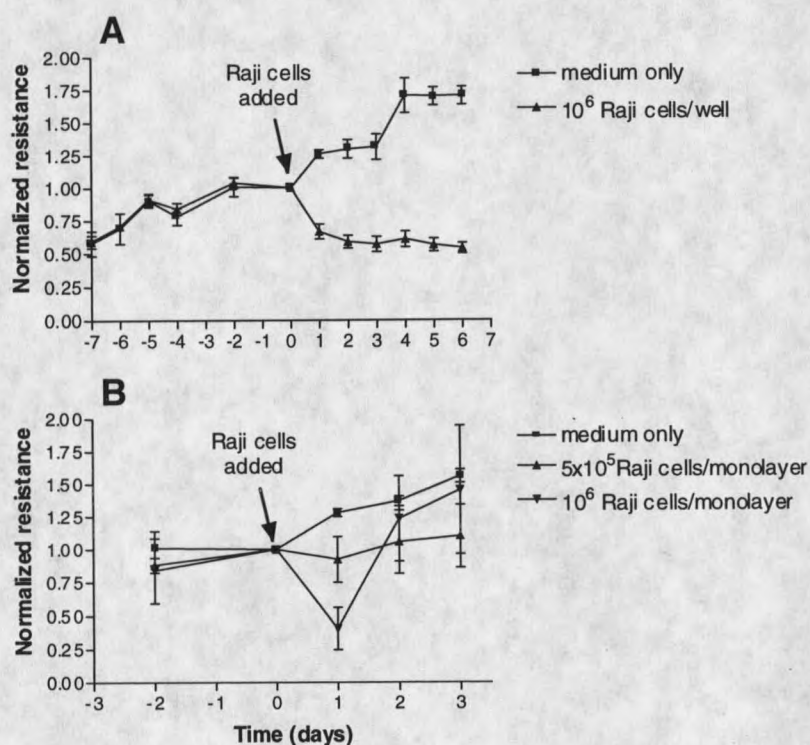


Figure 16. Transepithelial Electrical Resistance of GI Epithelial Monolayers after the Addition of Raji Cells. Raji cells were added to the basolateral side of Caco2 (A) or T84 (B) monolayers and the transepithelial electric resistance was measured. Monolayer resistances were normalized to 1 for $t = 0$ h. Figures A and B are representative examples from at least 3 separate experiments. Samples were run in triplicate. Error bars indicate standard deviation.

Immunofluorescence Microscopy

It has been reported that human M cells preferentially expressed the sialyl Le^a antigen on their surface. Therefore, we used an anti-sialyl Le^a antigen monoclonal IgG₁, KM231, to determine the expression of this antigen on Caco2 and T84 monolayers. No fluorescence was observed with Caco2 monolayers, but T84 monolayers were strongly reactive with KM231 (data not shown), suggesting T84 cells expressed the sialyl Le^a antigen on their surface. This indicated that we would be unable to use the expression of sialyl Le^a antigen as a marker for M cell production.

Cytochemical analysis of M cells of murine Peyer's patches suggested that these cells displayed a markedly reduced expression of alkaline phosphatase activity on their apical surface. We attempted to determine the alkaline phosphatase activity of Caco2 and T84 monolayers using a commercial endophosphatase detection system that is based on the production of a fluorescent precipitant at the site of enzyme activity. Both Caco2 and T84 monolayers displayed endogenous alkaline phosphatase activity (data not shown). Unfortunately, the staining pattern was not uniform and the precipitant formed small crystal-like patterns that made it difficult to distinguish M cells with decreased alkaline phosphatase activity from enterocytes.

Discussion

It initially appeared that *C. albicans* yeast stimulated an increase in transepithelial electrical resistance of T84 monolayers. Further analysis indicated that the increased resistance was most likely due to the acidification of the medium by *C. albicans*. The monolayers were very sensitive to a slight change in the pH of the medium, as small as 0.2 pH units (Fig. 16). The transepithelial electrical resistance is a measure of the resistance to passive ion flow across the monolayer and is dependent upon the formation of intercellular tight junctions at the apical end of the lateral membrane (88). How the

altered pH stimulated an increase in the electrical resistance is unknown. As far as we are aware, this correlation has not been previously reported in the literature. Tyrosine phosphorylation of tight junction proteins, such as zonula occludens-1, and association with the actin cytoskeleton have been shown to be involved in tight junction formation (344). It is possible that these processes were stimulated by the decreased pH of the medium.

Unfortunately, we were unable to duplicate the *in vitro* system developed by Kernéis and colleagues (194). They used Peyer's patch cells and Raji cells to induce Caco2 enterocytes to switch to a M cell phenotype. Both cell lines available for our use had been in culture for extensive periods of time. Therefore, it is possible that the Caco2 and Raji cell lines we were using had lost some of the characteristics necessary for the induction of M cells. In addition, the Raji cells seemed to have an adverse effect on the Caco2 monolayers. It was reported that Raji cells, 10^6 per monolayer, did not effect the transepithelial electrical resistance (194). However, we observed a significant decrease in Caco2 monolayer resistance after the addition of Raji cells (Fig. 17A) that seemed to correspond to detachment of Caco2 cells from the porous support. Therefore, we attempted to use T84 cell monolayers to induce M cells. Caco2 and T84 cell lines are both human colonic epithelial cell lines that have been shown to display phenotypic characteristics of and functional similarity to native intestinal crypt epithelial cells (88,163,239). Although, these cell types do differ in surface antigen expression of sialyl Le^a (data not shown) and T84 monolayers achieve higher transepithelial electrical resistance than Caco2 monolayers (personal observation). Similar to Caco2 monolayers, the Raji cells seemed to have an adverse effect on the T84 monolayers. After the addition of Raji cells, the resistances of the monolayers dropped (Fig. 17B). The drop in resistance was dependent upon the dose of Raji cells (Fig. 17B), although, the degree in resistance decrease was variable from experiment to experiment. The decrease in

resistance may have been due to dissociation of some T84 cells from the porous support or an effect directly on the transcellular tight junctions. T84 cells do display greater adherence avidity to collagen coated tissue culture wells than Caco2 cells (personal observation), and this may explain why the T84 monolayers did not dissociate from the porous support after the addition of the Raji cells. The T84-Raji cell co-cultures did regain a high resistance after 24 to 72 h post Raji cells addition. A slight increase in adhesion by *C. albicans* yeast to T84-Raji co-cultures was observed, although it was not significant. We were unable to determine if M cells were produced in these monolayers. Therefore, these results do not support or reject the hypothesis that M cells transport *C. albicans* yeast across the GI mucosa.

Studies suggest *C. albicans* expresses a fucose-specific adhesin that may be involved in adherence to epithelial cells (56,403,410). Sialyl Le^a antigen is a fucose containing glycoside. Human M cells have been shown to up-regulate the expression of sialyl Le^a (142). It is possible that *C. albicans* may recognize M cells through expression of a fucose-specific adhesin. In addition, it was intriguing that T84 and Caco2 monolayers differed in expression of sialyl Le^a antigen. Therefore, it is interesting to speculate on the role of sialyl Le^a in *C. albicans* adhesion to the T84 and Caco2 monolayers shown in Chapter 3; figure 10. It is possible that *C. albicans* was more adherent to T84 monolayers, but not to Caco2 monolayers, compared to *C. glabrata* (Fig. 10), because of the recognition of sialyl Le^a by the fucose-specific adhesin.

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