

Macrophages and interferon gamma in host defense against disseminated candidiasis
by Qinfang Qian

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:
To evaluate the role of macrophages in experimental disseminated candidiasis, mouse splenic macrophages were eliminated by intravenous delivery of liposome-entrapped dichloromethylene diphosphonate (L-Cl2MDP). Splenic tissue sections immunoperoxidase stained with monoclonal antibodies against marginal zone macrophages, red pulp macrophages and neutrophils showed that 3 days after L-Cl2MDP treatment, macrophages but not neutrophils were depleted, and circulating neutrophils responded normally to an irritated peritoneum and showed normal phagocytic and killing ability to C. albicans. The spleens from L-Cl2MDP-treated mice lost their ability to bind yeasts, which agrees with our previous findings that yeast cells bind specifically to marginal zone macrophages. When macrophage-depleted mice were given intravenously with C. albicans, the clearance of Candida from blood in these mice was slower, their kidneys had higher recoverable CFU, and they did not survive as long as control mice. These results indicate that macrophages play an important role in host resistance to experimental disseminated candidiasis, but the mechanism does not appear to involve thymus derived T-cell functions.

IFN-γ gene knock-out (GKO) mice were used to evaluate the role of interferon gamma (IFN-γ) in host defense against disseminated candidiasis. Genotypes of mice were determined by PCR, and were confirmed by ELISA, which showed no detectable IFN-γ produced by their splenocytes. GKO mice infected intravenously with C. albicans survived as long as wild type (WT) mice did and showed no difference in Candida CPU from different organs as compared to controls. When animals were given Candida intragastrically, there was no fungal dissemination to different organs from GKO or WT mice. Candida CPU recovered from the stomach or intestines of GKO and WT mice did not differ. GKO mice did not show evidence of more tissue damage or fungal invasion in the stomach cardial-atrium fold, where the fungus was located, than WT mice. Finally, the jejunum of both types of mice showed no evidence of fungal invasion. Although, IFN-γ is critical for Candida induced LA antigen expression by peritoneal macrophages, IFN-γ is not essential in host defense against disseminated candidiasis in mice.

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APPROVAL

of a thesis submitted by

Qinfang Qian

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Jim E. Cutler _____ 4/24/97
Date

Approved for the Department of Microbiology

Keith Cooksey _____ 4/24/97
Date

Approved for the College of Graduate Studies

Robert Brown _____ 4/27/97
Date

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## ABSTRACT

To evaluate the role of macrophages in experimental disseminated candidiasis, mouse splenic macrophages were eliminated by intravenous delivery of liposome-entrapped dichloromethylene diphosphonate (L-$Cl_2$MDP). Splenic tissue sections immunoperoxidase stained with monoclonal antibodies against marginal zone macrophages, red pulp macrophages and neutrophils showed that 3 days after L-$Cl_2$MDP treatment, macrophages but not neutrophils were depleted, and circulating neutrophils responded normally to an irritated peritoneum and showed normal phagocytic and killing ability to *C. albicans*. The spleens from L-$Cl_2$MDP-treated mice lost their ability to bind yeasts, which agrees with our previous findings that yeast cells bind specifically to marginal zone macrophages. When macrophage-depleted mice were given intravenously with *C. albicans*, the clearance of *Candida* from blood in these mice was slower, their kidneys had higher recoverable CFU, and they did not survive as long as control mice. These results indicate that macrophages play an important role in host resistance to experimental disseminated candidiasis, but the mechanism does not appear to involve thymus derived T-cell functions.

IFN-γ gene knock-out (GKO) mice were used to evaluate the role of interferon gamma (IFN-γ) in host defense against disseminated candidiasis. Genotypes of mice were determined by PCR, and were confirmed by ELISA, which showed no detectable IFN-γ produced by their splenocytes. GKO mice infected intravenously with *C. albicans* survived as long as wild type (WT) mice did and showed no difference in *Candida* CFU from different organs as compared to controls. When animals were given *Candida* intragastrically, there was no fungal dissemination to different organs from GKO or WT mice. *Candida* CFU recovered from the stomach or intestines of GKO and WT mice did not differ. GKO mice did not show evidence of more tissue damage or fungal invasion in the stomach cardial-atrium fold, where the fungus was located, than WT mice. Finally, the jejunum of both types of mice showed no evidence of fungal invasion. Although, IFN-γ is critical for *Candida* induced IA antigen expression by peritoneal macrophages, IFN-γ is not essential in host defense against disseminated candidiasis in mice.

# Chapter 1

## INTRODUCTION

*Candida albicans* is an opportunistic fungal pathogen of humans. It is a member of the normal flora of the human mouth (238), gastrointestinal (GI) tract (52) and vagina (181). *C. albicans* is a polymorphic fungus which can grow as yeast, pseudohyphal and hyphal forms in vitro (199) and in vivo (6,16). Candidiasis has risen dramatically over the last two decades. *Candida* species are now the fourth leading cause of all blood stream and urinary tract nosocomial infections in the United States hospitals, and they are the leading cause of fungal diseases in hospitalized patients (122). Although other *Candida* species, such as *C. tropicalis*, *C. parapsilosis*, *C. glabrata* and *C. krusei* also cause diseases, *C. albicans* is the major cause of candidiasis (73,121).

Candidiasis has become a costly disease nationally and is the cause of significant suffering and death. The number of antifungal drug choices has increased in recent years, but, as discussed below, all have serious short-comings in the treatment of this disease. An understanding of host defense mechanisms against *Candida* may well lead to improved methods of prevention and treatment of the disease through immune therapy. The immune response to *Candida* invasion is, however, very complex and defense mechanisms are still not entirely understood. My dissertation has focused on the role of macrophages and interferon-gamma (IFN-γ) in host defense against disseminated candidiasis. This

introduction will give an overview of the current level of understanding of *Candida*-host interactions. I will discuss predisposing factors and clinical aspects of candidiasis, treatment of the disease, host defense mechanisms against candidiasis, and proposed candidacidal mechanisms of macrophages and/or monocytes.

## Predisposing Factors and Clinical Aspects of Candidiasis

The increasing incidence of candidiasis is mainly because of the increasing number of immunocompromised patients. The latter increase is due to AIDS, chemotherapy against malignancies, certain kinds of cancer, immunosuppression of patients receiving organ transplants, treatment with broad spectrum antibiotics (136,227), the use of invasive devices and procedures such as central venous catheters (224) and the use of total parenteral nutrition (176,268).

*Candida* species can cause mucocutaneous and disseminated forms of candidiasis. Different factors predispose patients to different forms of the disease. Patients with AIDS usually develop severe mucosal candidiasis including oropharyngeal or esophageal candidiasis (261) and *Candida* vaginitis (213). Until recently, these patients rarely developed invasive candidiasis (about 3%) (85). However, the rate of disseminated candidiasis in these patients is increasing concomitantly with the use of anti-HIV medicines (ganciclovin and zidovudine) and antineoplastic agents which induce neutropenia in 13-20% of these patients, and the increased use of central venous catheters (95). Patients with disseminated candidiasis often have mucocutaneous candidiasis, especially in the GI tract

(89,172). In one study, 94% of disseminated candidiasis patients had severe GI candidiasis involving all segments of the GI tract, but most commonly the esophagus and stomach (89).

Patients with candidemia may develop disseminated disease (about 50% of patients) (172), which may involve the liver, spleen, kidneys, lung, brain, heart, and other organs (112,172,251). Acute lymphocytic leukemia was reported as the most common underlying disease in patients with candidemia (227), and candidemia occurred in 40-45% of bone marrow transplant patients (107). In addition, epidemiologic analysis showed that intravascular catheterization and total parenteral nutrition were significantly associated with candidemia (17).

Neutropenia, which may be caused by drugs or diseases, is also a predisposing factor to disseminated candidiasis (227). Neutropenia is claimed to be the most important predictor for surviving of patients with disseminated candidiasis (172).

Preterm infants and surgical patients (11,19,127,258) are vulnerable to disseminated candidiasis. The incidence of this form of candidiasis in very low birth weight infants (<1500g) is 3-5%, but in extremely low birth weight infants (<800g), the incidence is 18-20% (11). Surgical patients are predisposed to disseminated candidiasis, especially those patients with multiple operations or abdominal operations (176). The underlying diseases, such as malignancies and diabetes, and medical treatments, such as broad-spectrum antibiotics and prolonged catheterizations (19,176) may also predispose surgical patients to the disease.

Treatment with broad-spectrum antibiotics, especially vancomycin and/or imipenem predisposes host to disseminated candidiasis (227). Treatment of mice with antibiotics promotes GI colonization with *C. albicans* in mice, and the strictly anaerobic bacterial normal flora appear to inhibit *Candida* adhesion, colonization and dissemination from the GI tract (136). In agreement with the study in mice, in patients who had acute lymphocytic leukemia, 67% of those who received vancomycin (oral or intravenously) had a greater than four log increase in numbers of *Candida* in the stool (227).

Surprisingly, only the use of broad-spectrum antibiotics has been shown to be an independent risk factor for disseminated candidiasis. Other factors, such as total parenteral nutrition and steroids, were significant only when combined with antibiotic therapy (176,227). Neutropenic patients typically receive high doses of broad-spectrum antibiotic (24). These observations suggest that for prevention of disseminated candidiasis the kind of broad-spectrum antibiotic must be selected carefully to avoid significant disturbance of strict anaerobic bacterial normal flora in the GI tract. If antibiotics that disturb the anaerobic flora must be used, the patient may also should receive antifungal agents to prevent the development of disseminated candidiasis.

*C. albicans*, which causes the disease, could be of endogenous normal flora (108) or exogenous origin via wounds or catheters (16). Generally, invasive candidiasis is caused by endogenous colonized *Candida*, resulting from the 'escape' of the colonizing organism from host control (108). Colonizing and invasive isolates from the same patient may have different colonial morphologies, but DNA analysis indicates that the invasive form may well

have derived from the colonizing strain (263,298). Ulceration and damage to the epithelium from chemotherapy and coexistent infection with other microorganisms may facilitate the escape (212).

Nosocomial candidemia is both deadly and costly. The mortality rate in bone marrow transplant patients with candidemia is 73-90% (186), in extremely low birth weight infants the mortality rate is 20-55% (127). About one-half of cancer patients with candidemia will die of the fungal disease (172), and in patients with purulent pericarditis caused by *Candida* species, there is about a 54% mortality rate (251). Patients who survive candidemia will spend about 30 additional days in the hospital as compared with patients with similar underlying conditions but who did not develop candidemia (268,299).

## Treatment of Disseminated Candidiasis

The management of disseminated candidiasis in immunocompromised patients is difficult. Treatment with antifungal agents is not satisfying and immune therapy is only at an experimental stage. There are three different kinds of antifungal drugs with systemic activity: amphotericin B (a polyene), 5-fluorocytosine, and azoles. These agents tend to cause either severe side effects or resistant fungi tend to emerge.

Although amphotericin B is fungicidal in vitro and has been the drug of choice for invasive candidiasis (183), in vivo effectiveness and side effects are major concerns. Despite high concentrations of amphotericin B (200 mg/L) in tissues, failure to clear the fungus has been reported (56). The mortality rate is 30-60% of patients with disseminated candidiasis

even with amphotericin B treatment (185,227). With the increasing extensive prophylactic use of this polyene, resistant strains of the fungi are emerging (78,184). Side effects are multiple and include headache, chills, fever, nausea, vomiting, diarrhea, anorexia, malaise, muscle and joint pain, phlebitis at the infusion site, hypokalemia, anemia, bronchospasm, arrhythmia, and liver and kidney toxicity (240,277). As an additional complexity, amphotericin B and the immunosuppressive drug cyclosporin, which is used in transplant patients, are synergistic in causing nephrotoxicity (253).

Recently, less toxic forms of amphotericin B have been made, such as liposomal amphotericin B (L-AmpB) (28). The mechanisms of L-AmpB action are not well understood. They appear to be related to tissue targeting, altered interactions between the fungi and mammalian cells, and possibly to intracellular delivery of the drug to phagocytic cells (168). Autopsy studies of three patients treated with L-AmpB indicate that amphotericin B concentrated in liver and spleen (229). This suggested that L-AmpB might be entrapped in the organs of the reticuloendothelial system. In vitro studies showed that amphotericin B has immunoadjuvant properties. It stimulates B lymphocytes and increases phagocytosis, interleukin-1 secretion and superoxide anion production by macrophages in culture (22,165,302). Amphotericin B also stimulated immune responses in vivo (166). These observations suggest that mechanisms of L-AmpB against fungal infection may involve: (1) delivery of the drug directly into macrophages which may have already phagocytosed the yeasts, and (2) delivery of the drug to macrophages which results in enhanced phagocytic cell anti-C*andida* activity. L-AmpB also has side effects on patients,

including renal impairment (252), mild hypokalemia (168), and elevated levels of one or more liver enzyme (218).

Although 5-fluorocytosine (flucytosine) is effective against *Candida* infection, fungal resistance and drug side effects are problems. The emergence of drug-resistant strains is a major problem, especially when the drug is used alone (10,58,148). Furthermore, in one study, 20 % of *Candida albicans* from over 400 *Candida* isolates were naturally resistant to this drug (264). Flucytosine is used as adjunctive therapy with amphotericin B, although the effectiveness of such adjunctive therapy has been debated (36). When flucytosine is used alone, side effects occur only in a small portion of patients. When the drug is used in combination with amphotericin B, toxic effect occurred in 15-30% of patients (147). Potentially dangerous side effects of flucytosine include bone marrow suppression and liver function impairment (250).

Azoles are treatment alternatives to amphotericin B because they are easily administered and have fewer side effects. Azoles are generally fungistatic rather than fungicidal (286). The azole drugs commonly used for treating disseminated candidiasis are ketoconazole, fluconazole, and itraconazole (196,197,278,280). The problem with azoles is the emergence of drug-resistant strains of *C. albicans* and natural resistance of other *Candida* species such as *C. krusei* and *C. glabrata* (200,303).

## Host Defense Mechanisms against Candidiasis

The increasing clinical importance of *Candida* species and treatment dilemmas associated with candidiasis are reasons to gain an understanding of the host defense mechanisms against *Candida.* This may well lead to better treatment and prevention of candidiasis through immune therapy.

Unlike other species of *Candida*, *C. albicans* is a member of the normal flora of humans with whom it enjoys a commensal relationship. As alluded to earlier, disseminated candidiasis is often caused by *C. albicans* from an endogenous location, such as the GI tract. A combination of several interactive innate and specific immune defenses appears to be required for optimal expression of anti-*Candida* resistance that prevents the disease.

### Innate Nonspecific Immune Mechanisms Against Candidiasis

Microbes of the normal flora, which are commensal to the host, are considered as a part of the host innate defense mechanisms. Strictly anaerobic bacteria in the GI tract inhibit *C. albicans* adhesion, colonization and overgrowth possibly as a result of competition for nutrients, blocking of receptor sites on epithelial cells or fungal inhibitory factors. In one study, depletion of these normal residents by treatment of mice with clindamycin, penicillin or vancomycin resulted in increased susceptibility to disseminated candidiasis of endogenous origin (136). Observations on patients with disseminated candidiasis also supports the above study (227).

The host surface, the skin and mucosal membranes, is a physical barrier against infectious microbes and constitutes an essential first line of the immune defense system. The integrity of epithelial cells is important in separating the host's internal milieu from the external environment. Continuous shedding of mucosal epithelial cells may play a role in limiting surface adherence and invasion of the fungus (129).

Interestingly, mucosal epithelial cells are not just functioning as a physical barrier; they also seem to play an active immunological role, such as cytokine production, in host defense against microbial invasion. Although little information on pathogenic fungi is available on this topic, studies of bacteria showed that invasion of *Escherichia coli* into colon epithelial cell lines induced production of interleukin 8 (IL-8), monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor α (TNF-α) and granulocyte-macrophage colony stimulating factor (GM-CSF) (129). IL-8 and MCP-1 are potent chemoattractants and activators for neutrophils and monocytes, respectively. TNF-α activates neutrophils and mononuclear phagocytes, GM-CSF prolongs the survival of neutrophils and monocytes, and both TNF-α and GM-CSF increase neutrophils' and monocytes' anti-*Candida* activity (23). Freshly isolated intestinal epithelial cells produced IL-6, which is a B cell proliferating factor (144), and this production was up regulated in response to bacterial invasion (129). Damaged epithelial cells released preformed IL-1, which stimulated other viable epithelial cells to secrete IL-8 (86). Bacterial invasion of colonic epithelial cells up regulates the expression of adhesion molecules on epithelial cells and promotes neutrophil adherence to infected cells (116). These observations suggest that intestinal epithelial cells may play an

important role by providing early signals for the initiation of an acute mucosal inflammatory response. Although it is not known if *Candida* invasion causes the same effect, investigators found that *C. albicans* yeast cells invade columnar epithelial cells in vivo (54) and hyphae invade rabbit oral epithelial cells (115). However, these works are limited. It will be interesting to study if the interaction of the fungi with epithelial cells induces these cell produce cytokines which initiate an acute mucosal inflammatory response. Those cytokines, which enhance phagocytic cell anti-*Candida* activities, such as TNF-α and GM-CSF (23), may be important in early stages of fungal invasion to prevent the disseminated disease. In addition, squamous mucosal epithelial cells can release leukocyte protein L1, which can inhibit the growth of *C. albicans* (30).

Initial steps in host immune defense against disseminated candidiasis may involve non-specific serum factors, especially complement. No difference was seen between C4-deficient and normal control guinea pigs to the disseminated disease. However, C4-deficient guinea pigs treated with cobra venom factor (CVF), which depletes complement factors C3 to C9, were more susceptible to an intravenous (i.v.) dose of *C. albicans* than C4-deficient animals without CVF treatment and normal control animals. These C4-deficient, CVF treated guinea pigs did not survive as long as control animals did, and there were more *Candida* colony forming units (CFU) recovered from their kidneys than from that of control guinea pigs (105). C5-deficient mice are also more susceptible to systemic infection as indicated by CFU which develop in brain and kidney tissues of *Candida*-challenged mice (190). These data suggest that activation of the alternative pathway of complement is

important in host defense against disseminated candidiasis.

The mechanisms by which complement protects the host probably involve opsonization of the fungal elements and the recruitment of phagocytes to the infected sites (74,98). *C. albicans* can activate complement by the alternative pathway (142,219), which opsonizes the fungi and results in increased phagocytosis (191) and killing of the organism by neutrophils and macrophages (208,305). Intracellular killing of *C. albicans* by human neutrophils was not seen without C3 (305). This means that C3 is directly involved in the processes of phagocyte killing activity. In vitro, C5a complement factors are chemotactic to neutrophils, monocytes, basophils and eosinophils (74,98). Mice treated with CVF and congenital C5-deficient mice failed to induce a neutrophil inflammatory response to *Candida* in tissue (100,220). These data strongly suggest that complement protects the host against disseminated candidiasis by opsonization of the fungi and recruitment of inflammatory cells to the infected sites.

Other serum factors, such as iron-binding protein, mannose binding protein (MBP), and an unknown serum factor may also involved in host defense against disseminated candidiasis. Transferrin, an iron binding protein, inhibits replication of the fungi (193). Indirect evidence suggests that MBP may be important in host defense against disseminated candidiasis (266). MBP activates the classical complement pathway. Sequence analysis revealed that MBP is structurally similar to a complement factor C1q (271). This suggests that MBP mimics the C1q to activate the complement pathway. *Candida* has mannan which could be recognized by MBP (266). Serum MBP level drop about 39% of the initial level

followed by an i.v. injection of *C. albicans*. Eventually, the MBP could act as a universal antibody by binding to the fungi and activating the complement pathway resulting in the opsonization of the fungi. This process may increase the phagocytosis of *Candida* by phagocytes. The unknown serum factor exerts an inhibitory effect by causing yeast clumping (170,255), but its significance is not clear.

Studies on neutrophils in vitro (51,175), of animals with experimental systemic candidiasis (9,230), and of patients who develop disseminated candidiasis (292,300) strongly support the concept that neutrophils are critical in host immune defense against disseminated candidiasis. Neutrophils can phagocytose *Candida* yeast form cells, and kill both yeast and hyphae intracellularly or extracellularly. The killing mechanisms involve the NADPH oxidase , the myeloperoxidase-hydrogen peroxidase-halide system, the ferrous iron-hydrogen peroxide-iodide system, lysosomal enzymes, and defensins (51,71,75,76,79,85,102,159,169). Neutrophils have the highest candidacidal activity among leukocytes (155), and this property is enhanced by opsonization with antibody and complement (208,305). Moreover, dead neutrophils release a 30 kDa calcium-binding protein (calprotectin), which is candidastatic, as another way to inhibit the fungus (182,241,257). Mice treated with mAb to deplete granulocytes are dramatically more susceptible to an i.v. challenge with yeasts (110,124). In agreement with the above observations, mice made neutropenic by cyclophosphamide treatment had increased susceptibility to an i.v. challenge of *Candida* (145). Severity of neutropenia in patients correlates with susceptibility to disseminated disease (4,137).

Although there is strong evidence that neutrophils are important in host defense against disseminated candidiasis, the role of neutrophils in preventing spread of the fungus from a mucosal site is not clear. Tissue histology studies give evidence that neutrophils accumulate at the mucosal site where *C. albicans* invaded. When neonatal mice were given a large number of *Candida* yeast cells ($10^8$) intragastrically (i.g.), intraepithelial abscesses developed in the stomach mucosa. Both hyphae and numerous neutrophils were present at the site of infection abscess [ (53) and our unpublished observations]. Normally, neutrophils are not present in high numbers in tissue, but they may be attracted to the infected tissue by chemokines (201), complement components (98), or chemotactic factors produced by the fungus itself (31,32,62) and activated by activating factors. Oropharyngeal candidiasis is a common infection among cancer patients, and especially during neutropenic periods caused by the chemotherapy and/or the cancer (70). However, mice that were made neutropenic by treatment with cyclophosphamide showed no dissemination of *Candida* via the GI tract (287). More studies are needed to reveal the role of neutrophils in the mucosal site against candidiasis.

The ability of macrophages to phagocytose and kill *Candida* strongly suggests that macrophages are important in host defense against disseminated candidiasis. Murine peritoneal macrophages (35,222), pulmonary alveolar macrophages (244), Kupffer cells (245,246) and human peripheral blood monocytes (120,173) can ingest and kill *C. albicans*. Non-activated macrophages, such as rabbit resident peritoneal and alveolar macrophages, had relatively low candidacidal activities. Unstimulated human monocytes could even be killed

by phagocytosed *Candida* yeast cells, which germinated and penetrated cell membranes within monocytes (68). Mouse and rabbit macrophages were also killed by intracellular proliferation of *Candida* (5,90). Activated macrophages are more efficient in phagocytosis and killing of the fungi. Murine and human macrophages and human monocytes, which were activated in vitro, had increased ability to ingest and kill *Candida* (35,113,295), about 2-3 times more efficiently than resident macrophages (242). Enhanced killing in vitro was also observed when cells were activated in vivo by treatment of the animal with complete Freud's adjuvant (156). Activated peritoneal macrophages from *Listeria*-immunized mice were found to be able to kill *Candida* hyphae extracellularly (113). All of these studies suggested that in cooperation with other host cells, which produce macrophage-activating cytokines, macrophages may be an active force against disseminated candidiasis.

Histopathological studies in animals also gave indirect evidence that macrophages may be important in host defense against disseminated candidiasis. Infiltration of *Candida*-infected tissue by macrophages has been observed (207,231). When mice were given *Candida* yeast cells i.v., the majority of the fungus were found in the liver and lungs, yet these organs rarely sustained even minor tissue damage (7,204). Alveolar macrophages and Kupffer cells may clear the fungi from these organs.

Although the above observations suggest that macrophages may be important in the host defense against disseminated candidiasis, in vivo studies yield controversial results (table 1). In some studies, nonspecific activation of macrophages by either *Bacillus* Calmette-Guerin (BCG) (8) or by a poorly virulent agerminative strain of *C. albicans* (strain

PCA-2) (20) enhanced the resistance of mice to disseminated candidiasis (8). Consistent with these observations, an in vivo administration of a macrophage activation factor, IFN-γ, resulted in increased protection of mice against this disease (222). Transfer of splenic macrophages from *Candida*-immunized mice to naive mice enhanced their resistance to the disease (20). Others found, however, that in vivo administration of BCG did not increase the resistance of mice to disseminated disease (230). In vivo administration of murine recombinant IFN-γ to mice either increased the susceptibility of mice to the disease (103), or the protective effect of IFN-γ was attributed to neutrophil activation (146). It was even suggested that macrophages play a major role in the pathology of disseminated disease. When mice were given macrophage colony stimulating factor (M-CSF), which elevated circulating monocytes and tissue macrophages, they were more susceptible to the disease (118). Furthermore, intravenous administration of the macrophage toxin silica into normal mice increased their resistance to disseminated candidiasis (152), but this effect did not occur in *nu/nu* mice (152), *bg/bg* mice or their normal controls (8). The limitation of silica treatment is that it can impair both neutrophil and macrophage functions (307), and silica may cause macrophages to produce cytokines, such as IL-1 (259). The lack of a method to specifically deplete macrophages is a major reason for the inconclusive data. Different experimental design, such as routes and doses of the agents given to the animal, may also account for the controversial results. Further studies are required to clarify if macrophages play a protective role in host defense against this disease.

Table 1. Controversy of the role of macrophages in host defense against disseminated candidiasis in mice

| | |
|---|---|
| Supportive | 1986 Transfer of splenic macrophages from *C. albicans* immunized mice increase naive mice resistance (20)<br>1988 Activation of macrophages by BCG enhanced resistance (8)<br>1993 Administration of IFN-γ increased resistance (222) |
| Against | 1977 BCG administration did not increase resistance (230)<br>1983 Administration of macrophage toxin silica increased the resistance of BALB/c mice, but not *nu/nu* mice (152) and *bg/bg* mice (8)<br>1989 Administration of IFN-γ increased the susceptibility (103)<br>1992 M-CSF enhanced mouse susceptibility, macrophages play a role in the pathogenesis of disseminated candidiasis (118)<br>1993 Administration of IFN-γ protect mice, but it is due to neutrophil activation (146) |

Macrophages may also be important in preventing the spreading of *Candida* from mucosal site. In vitro, macrophages can kill both yeasts and hyphae that have been either ingested or remain outside of the phagocyte (113,222). An immunohistochemical study (205) revealed that macrophages accumulate as a thick band immediately beneath the human colon epithelium. Further, *bg/bg* mice were more susceptible to GI colonization, and *bg/bg-nu/nu* mice were more susceptible to mucosal and systemic candidiasis of endogenous origin (37). The *bg/bg* defect involved not only neutrophils but also macrophages (38). It is possible that macrophages play a role in host defense against candidiasis at the mucosal site. Furthermore, studies on mice with severe combined immunodeficiency (*scid*) gave indirect evidence that mucosal macrophages may contribute to the resistance of these mice to orogastric candidiasis. *C. albicans* monoassociated *scid* mice treated with nitric oxide

synthase inhibitor (iNOS) developed more severe mucosal candidiasis than control mice did. An increase in the iNOS mRNA was observed in gastric tissue of *Candida* monoassociated *scid* mice (291). Murine macrophages (249), but not murine neutrophils express iNOS and produce high amounts of NO, which was shown to be related to macrophage candidacidal activity (288). Please refer to the section on candidacidal mechanisms of macrophages/monocyte below for further discussion on NO.

Possible mechanisms by which macrophages could protect the host against disseminated candidiasis from a mucosal site may include direct anti-*Candida* activity via phagocytosis and killing of the fungus, or indirect activity via cytokine production, such as, IL-1 and TNF-α (84). These cytokines enhance other cells, such as neutrophils, anti-*Candida* activity (83,91). A combination of T cell and macrophage deficiency may also be responsible for the predisposition of *bg/bg-nu/nu* mice to the disseminated disease, because these mice are deficient in not only neutrophils and NK cells, but also T cells and macrophages (see discussion in T cell section below).

Although not well studied, there is some indirect evidence suggest that natural killer (NK) cells may be involved in the host defense against disseminated candidiasis. NK cells do not kill *Candida* directly (308), yet NK cells stimulated by *C. albicans* produce cytokines, including IL-8, TNF-α, IFN-γ and GM-CSF. These cytokines may attract phagocytes to the infected sites and increase their candidacidal activities (21,82,84,203,274). When mice were given merthiolate inactivated *Candida* yeast cells intraperitoneally (i.p.), NK cells were found to be recruited to the peritoneal cavity. When mice were given *Candida* i.v., splenic

NK activity was significantly increased (248). The best characterized immune deficiencies of neonatal mice relate to the deficiency of IFN-γ production by T cells and of NK activity (301). Infant mice developed systemic candidiasis if they were given i.g. $10^8$ *Candida* yeast cells (54,94). Similarly, mucocutaneous and disseminated candidiasis is a problem in human neonates (92). In addition to having deficient neutrophils and macrophages, *Bg/bg* mice have deficient NK cells (38). *Bg/bg-nu/nu* mice were more susceptible to disseminated candidiasis of endogenous origin, and *bg/bg* mice were more susceptible to GI colonization (37). A low NK activity in vivo may correlate with the development of disseminated candidiasis in infants.

## Specific Immune Mechanisms Against Candidiasis

The role of antibodies in host defense against disseminated candidiasis has long been a controversial issue, but recent studies strongly suggest that antibody has protective effects against this disease. *Scid* mice, which lack of normal B lymphocytes, are not more susceptible to an i.v. inoculation with *Candida* than controls (171). Passive transfer of antibodies against *Candida* did not protect mice in one study (15). Almost all humans have antibodies to *C. albicans* in their sera owing to mucocutaneous (mainly GI tract) colonization (118,297). Patients with systemic *Candida* infection usually have specific antibodies in their sera, which suggest that these patients were not deficient in antibody production (85). All of these data suggest that antibodies may not play a role in host defense against disseminated candidiasis. However, some studies do show that antibody may play a protective role against

this disease. The earliest study was by Mourad and Friedman who showed that anti-*Candida* antisera significantly increased the resistance of naive mice to an i.v. challenge of *C. albicans* (192). Several other studies also showed that immune sera transferred protection against *Candida* (132,206). Matthews, et al. (177) demonstrated that antibody to an immunodominant 47 kDa *Candida* antigen protected against invasive candidiasis, which originates from GI colonization of patients with chronic mucocutaneous candidiasis. Passive administration of a specific antibody to an immunosuppressive B cell mitogenic protein, P43, which is produced by *C. albicans*, significantly protected mice against i.p. inoculation with the fungus. However, antibodies to *C. albicans* sonicates markedly increased the susceptibility of mice to the infection (267). The strong evidence for the protective activity of antibody to *C. albicans* is provided by Han and Cutler (109,110). They found that both polyclonal sera and a monoclonal antibody to a mannan adhesin fraction of *Candida* increase the resistance of mice to an i.v. challenge with the fungus. Two IgM monoclonal antibodies to *C. albicans* surface determinants were isolated that agglutinated *Candida* yeast cells, but only one of the antibodies protected mice. These studies suggest that different antibody specificity may explain the contradictory results. To achieve protection from disseminated candidiasis by antibody, the host must generate antibodies against specific kinds of epitopes/molecules of the fungus.

The role of antibody, especially secretory IgA (sIgA), in mucosal defense against candidiasis is not well understood. Data from several studies tend to go against the hypothesis that antibodies are protective. Non-specific sIgA enhanced adherence of *C.*

*albicans* to epithelial cells (294), and levels of IgA and IgG to *C. albicans* in vaginal fluid were similar in women with and without vaginal candidiasis (25). In agreement with the above data, an increase in sIgA specific to *C. albicans* in parotid and in whole saliva of both HIV-infected and AIDS patients was seen. This suggests that humoral immune responses are functioning in these people and development of mucosal candidiasis is not due to the deficiency of sIgA (57). Despite these negative reports, some studies imply that sIgA may protect the host. Two studies showed that specific sIgA inhibited the adherence of *Candida* to oral epithelial cells (88,275), but these studies were limited in samples and lacked proper controls. Increased sIgA levels to *Candida* were seen in Swiss and BALB/c mice after oral immunization with the fungus (188,217), but direct evidence for the relation between lower CFU in the saliva and the higher titer of specific sIgA was not studied in this work. When immunoglobulin (Ig) were harvested from vaginal fluid of *C. albicans*-immunized rats and were given to normal recipient rats intravaginally, the recipients had much less *Candida* burden in vagina than control mice when mice were given the fungus intravaginally. This effect did not happen in mice whom were given the non-Ig fraction of the vaginal fluid from immunized mice. When the Ig fraction was adsorbed with heat-inactivated *C. albicans* yeast cells, the protective effect was greatly reduced. Preadsorbing the Ig fraction with heat-inactivated *S. cerevisiae* did not affect the protection (39). This suggests that anti-*Candida* Ig contributes to the resistance of naive rats to vaginal candidiasis. However, it is not known if the protective effect is due to sIgA or other types of immunoglobulin, such as IgG or IgM.

$CD4^+$ T lymphocytes are believed to play a critical role in resistance to mucosal candidiasis. These cells reside at intraepithelial sites, the lamina propria, and within Peyer's patches (1,139). Gnotobiotic *nu/nu* (athymic) mice still had *Candida* hyphal invasion of their tongues and stomachs after 10 weeks of GI colonization, while nu/+ (phenotypic normal controls) mice cleared the disease by that time (13). *Scid* mice were more susceptible than normal controls to sustained GI colonization with *C. albicans*, and colonized *scid* mice had higher GI burdens of *C. albicans* than colonized normal mice (194). The limitation of these studies is that *scid* mice are deficient in not only T cells but also B cells. Elimination of $CD4^+$ T cells by treatment with anti-$CD4^+$ mAb abolished the ability of normal mice to clear mucosal candidiasis (38). These studies suggest that $CD4^+$ T cells play an important role in resistance of mice to mucosal candidiasis. This is in agreement with the observations of humans infected with HIV and of AIDS patients, who are deficient in $CD4^+$ T cells. About 50% of HIV-infected persons and 90% of AIDS patients have mucosal candidiasis (55,209).

The mechanism(s) of $CD4^+$ T cells in the mucosal defense against *Candida* is not known. One possibility is that these T cells produce cytokines, such as IFN-$\gamma$ which enhance phagocytic cell anti-*Candida* activity (77,83,174,262,274). When the host is deficient of T cells, phagocytes may not be fully activated without cytokines produced by these T cells, so that they are not as effective in killing the fungus as in normal mice (44,169). Other cells, such as NK cells (84,274), macrophages/monocytes (84), epithelial cells (129), or even neutrophils (202), produce cytokines, such as, IFN-$\gamma$, TNF-$\alpha$ or IL-1, which activate neutrophils. However, cytokine production by these cells may not be as potent and/or as

lasting as by T cells. Low levels of neutrophil activation and abnormal mucosal epithelium (see discussion below) may partly explain why AIDS patients develop mucosal candidiasis. Another possible mechanism is that in AIDS patients $CD4^+$ T cell deficiency may result in a relative deficiency of mucosal intraepithelial γ/δ T cells (see discussion below). IL-2 is the essential cytokine for activation and growth of these γ/δ T cells, and $CD4^+$ α/β intraepithelial cells are capable of producing IL-2 upon stimulation with TCR-CD3 complex (1).

The characteristics of γ/δ T cells suggest that this subgroup of T cells may also be important in host defense against candidiasis. These cells are found predominantly in the mucosal epithelium and in the lamina propria of small intestine (27). Intraepithelial γ/δ T cells modulate the growth and differentiation of GI epithelial cells (26). Disruption of the T cell receptor δ gene causes a reduction of epithelial cell turnover (141). Importantly, these T cells produce cytokines, such as IFN-γ and TNF-α (46), which increase anti-*Candida* activity of neutrophils and macrophages (23,77,81,174,226,256,262,295). Animal studies also suggested that γ/δ T cells may protect the host against this disease. These T cells increased in the gastric mucosa of germfree mice after GI colonization with *C. albicans,* and in the peritoneal cavity when mice were given *C. albicans* i.p. (128). This subgroup of T cells also increased in the basal epithelial layer and subepithelial areas of the oral mucosa and minor salivary glands when mice were given *Candida* orally (43). Interestingly, this recruitment peak coincides with a dramatic decrease of viable *Candida* in mucosal tissue (43).

Although T cells are critical in host defense against mucosal candidiasis, the importance of T cells in the host defense against disseminated candidiasis has long been a controversial issue. AIDS patients do not usually develop disseminated candidiasis, and animals deficient in T cells, such as nude mice and *scid* mice did not develop disseminated disease when colonized with the fungus (37,125). These mice were even more resistant than normal mice to an i.v. challenge with *Candida* (61,151,171,232). Compensatory mechanisms may be operating in these mice because nude mice are thought to have inherently activated macrophages (45) and they might have more neutrophils in their spleens than normal control mice (our unpublished data).

Recent studies indicate that T cells may be important against disseminated candidiasis. *C. albicans* monoassociated nu/+ mice cleared an i.v. inoculation of *C. albicans* faster than did *nu/nu* mice (12). A *C. albicans* specific $CD4^+$ T cell line protected mice against i.v. challenge of the fungus. When BALB/c mice were irradiated and then received the T cell line, they had fewer *Candida* CFU in their kidneys and lived longer than control mice which did not receive the T cells (254). Furthermore, treatment of mice with anti-Lyt2.2 mAb, which depletes T cytotoxic cells, greatly enhanced the susceptibility of mice to i.v. challenge with *Candida*. Treatment of mice with anti-Lyt2.2 and anti-L3T4 mAbs, which depletes both T helper cells and T cytotoxic cells, showed a dramatic increase in the susceptibility of mice to i.v. infection with *C. albicans* (42). These data indicate that both T helper cells and T cytotoxic cells play a role in host immune defense mechanisms against disseminated candidiasis.

Studies on multi-immunodeficient mice suggest that a combination of normal T cell and neutrophil functions may be critical in host defense against disseminated candidiasis that originates from a mucosal site. Twelve to sixteen weeks after *bg/bg-nu/nu* mice were given i.g. *Candida*, they developed extensive yeast and hyphal invasion of the palate, tongue, esophagus, and stomach, and had significantly higher numbers of CFU recovered from liver, spleen and kidneys than control mice (37). Depletion of circulating neutrophils with mAb to mouse granulocytes enhanced the susceptibility of *scid* mice, but not normal control mice, to disseminated candidiasis originating from a mucosal location (124). The limitations of the above studies are that those multi-immunodeficient mice have other immunodeficiencies along with the deficiency of T cells and neutrophils. In neutropenic patients, their mucosa may be damaged or altered by chemotherapy (212) or by treatments which impair T cell functions. On the other hand, *Candida* may overpopulate and colonize neutropenic patients because they routinely receive broad-spectrum antibiotics. These patients are at risk of developing disseminated candidiasis. This is consistent with the observation that patients who develop disseminated disease usually have multiple immunologic deficiencies.

Cytokine production by T cells may be a possible mechanism by which these cells protect against disseminated candidiasis. A Th1 response has been found to be associated with acquired resistance of mice to systemic candidiasis, and the protection is related to IFN-γ production (126,235). In support of this in vivo study, $Lyt2^+$ T cells produce IFN-γ in vitro in response to *Candida* antigen (42), and IFN-γ can enhance neutrophil and macrophage anti-*Candida* activity in vitro (77,174,262). IL-2 produced by T cells can activate $CD8^+$ T cell

to inhibit *C. albicans* hyphal growth in vitro (18). This fungal inhibititory ability by T cells may be another mechanism of T cell immunity against disseminated candidiasis in vivo.

Candidastatic/Candidacidal Mechanisms of Macrophages and/or Monocytes

Candidacidal activity of macrophages and/or monocytes is an important host defense against candidiasis. Understanding of the mechanisms of the killing activity may lead us to better treatment through enhancement of the antifungal activity of these cells. Macrophages from different anatomical sites are functionally heterogenous in anti-*Candida* activity. The candidastatic activity of macrophages freshly isolated from spleen was equivalent to that of Kupffer cells and alveolar macrophages, but peritoneal macrophages were much less effective (69). Activated macrophages or monocytes have increased anti-*Candida* activity. Monocytes and macrophages from naive animals have limited abilities to kill systemic mycotic agents when compared with cells activated with cytokines (34) or other microorganisms, such as *Listeria* (113). IFN-α/β (135), IFN-γ (23,195), TNF-α (23), GM-CSF (135,256) and IL-1 (23) have been shown to increase macrophage and monocyte candidacidal activities.

To exert the killing activity, macrophages or monocytes must first bind to their targets before phagocytosis and attempt to kill both intracellular and extracellular targets (113). Macrophages express receptors for IgG Fc, such as FcγRI, FcγRII, and FcγRIIIa (117), for complement C3b and C3bi, such as CR1 and CR3, respectively (143), and for mannose, which is in high concentration on the fungus (135). In contrast to macrophages,

the binding sites on human monocytes for unopsonized *Candida* yeast cells are the β-glucan receptors (120).

Macrophages or monocytes can exert their candidacidal activity by the production of toxic forms of oxygen. For the phagocytosis-associated respiratory burst, oxygen is converted to $O_2^-$ by NADPH oxidase (71,79,237). $O_2^-$ is relatively innocuous, but it is rapidly converted into $H_2O_2$. Furthermore, different pathways have been described by which microbicidal activities of $H_2O_2$ were increased. One requires myeloperoxidase (MPO), which catalyzes the peroxidation of $Cl^-$ to form HOCl (140,153,161). HOCl can react rapidly with primary amines or other nitrogen-containing compounds to yield derivatives containing the NCl bond (270). Monochloramine, produced by the reaction of HOCl with ammonium, is an example of such a powerful oxidizing agent generated by the MPO-$H_2O_2$-halide system (270). $H_2O_2$ can also be used by the iron-mediated Fenton and Haber-Weiss reactions to elicit toxic cell damage through generation of hydroxyl radical (99,161).

There is evidence which suggest that human blood monocytes possess two oxygen-dependent candidacidal mechanisms (154). One is identical to the MPO-$H_2O_2$-halide system of neutrophils, while the second is an MPO-independent system, which, in comparison, is less effective. The proposed existence of an MPO-independent oxidative candidacidal system in normal human monocyte is based on the observation that monocytes from a patient with MPO deficiency could kill *C. parapsilosis*, but monocyte from patients with chronic granulomatous disease could not. One possible explanation would be utilization of $H_2O_2$ by monocyte as well as macrophages in the iron-catalyzed Fenton and Haber-Weiss reactions

to produce hydroxyl radical. In this reaction, $O_2^-$ interacts with $Fe^{3+}$ to reduce the metal to $Fe^{2+}$. Then the interaction of $Fe^{2+}$ with $H_2O_2$ catalyzes the transfer of an electron from $Fe^{2+}$ to form $HO^{\cdot}$ (119,296). Freshly isolated human monocytes can ingest and kill *C. albicans*. When cultured in vitro for 3 days, monocytes could still ingest the fungus but lost their ability to kill *Candida*. Correlated with the reduction of candidacidal activity was a decrease in the activity of MPO and the production of hydroxyl radicals. However, the level of $O_2^-$ was similar regardless of the age of the monocytes. These data suggest that candidacidal activity of monocytes is associated with MPO and hydroxyl levels of the cell and not with superoxide generation (243).

Although macrophages can kill microorganisms via the oxygen-dependent NADPH oxidase pathway, they lack the MPO system for killing (50). A possible mechanism is comprised of ferrous ions, $H_2O_2$, the Fenton and Haber-Weiss reactions, which were found active against *C. albicans* yeast cells in vitro (161). Monocyte-derived macrophages kill opsonized *C. albicans* yeast cells and the killing was decreased by superoxide anion and hydrogen peroxide inhibitors (272).

Nitric oxide (NO) may also be involved in candidacidal activity of macrophages. NO may be an active intermediate of one of the microbicidal mechanism of activated macrophages (189). Murine macrophages synthesized NO upon appropriate stimulation, such as IFN-$\gamma$ and TNF-$\alpha$ (80). The killing of *C. albicans* by murine peritoneal macrophages activated by IFN-$\gamma$ was correlated with the production of NO by these cells (40). Higher nitrite production by peritoneal macrophages from *C. albicans* infected mice coincided

partially with the enhanced phagocytic and candidacidal activity. Addition of N-monomethyl-L-arginine (NMMA), an inhibitor of the nitric oxide synthase, inhibited both the nitric oxide production (99.6% inhibition) and the candidacidal activity (83.8% inhibition) (223). NO synthesized by inducible nitric oxide synthase may contribute to the resistance of *scid* mice to mucosal candidiasis (291). NO may not be directly candidacidal, but may be associated with other macrophage candidacidal mechanisms (289). Peroxynitrite ($ONOO^-$) a product of the reaction of NO and $O_2^-$, is a candidacidal molecule of activated macrophages (290). These data suggest that NO may be important in candidacidal activity of macrophages. It is not clear if NO is important in killing activities of human monocytes and macrophages because the role of NO production in human macrophages/monocytes is still controversial (72,211,306). However, there is evidence for the production of high levels of NO by human macrophages. Gp 120 HIV envelope glycoprotein induced high level of NO production by human monocyte-derived macrophages (211). Lipopolysaccharide induced human monocytes to produce NO (158). Further studies are required to establish if NO is involved in the candidacidal activity of human macrophages. Studies on the killing of bacteria suggest that macrophages from different anatomical sites have different killing abilities. NMMA inhibited the killing of *Francisella tularensis* by IFN-γ activated peritoneal macrophages, but this inhibition did not occur with IFN-γ activated alveolar macrophages (214). To study the role of NO in the candidacidal activity, the variations of macrophages need to be taken into account.

Monocytes and macrophages may also kill *Candida* via oxygen-independent mechanism(s) based on the following observations. (1) Intracellular killing was observed when these cells were incubated anaerobically (272). (2) Candidacidal activity by resident or elicited macrophages was only partially inhibited by scavengers of oxygen radicals (242). Monocyte and tissue macrophages also produce substances, such as leukocyte protein L1, a calcium binding protein (calprotectin), or cationic proteins to inhibit or kill *C. albicans* and other *Candida* species (29,30,66,157).

## Hypothesis

My hypothesis is that macrophages are important in host defense against disseminated candidiasis. In vitro, macrophages can phagocytose and kill *Candida* yeast cells and kill *Candida* hyphae extracelluarly, and produce cytokines, such as TNF-α, IL-1, which enhance neutrophil anti-*Candida* activity. The in vivo importance of macrophages in host resistance to the disease is controversial partly because it is difficult to specifically eliminate these cells from the host. In addition, in those studies attempting to address the role of macrophages, considerations of neutrophil functions are usually lacking.

In my dissertation work, I used a method for in vivo specific elimination of macrophages form the spleen and liver of mice to study the role of macrophages in host defense against disseminated candidiasis. For the depletion of macrophages, mice were given i.v. liposome-entrapped dichloromethylene diphosphonate (L-$Cl_2$MDP). $Cl_2$MDP is a cytolytic agent. When L-$Cl_2$MDP is in the circulation, macrophages will phagocytose and

disrupt these liposomes by phopholipase. Eventually the cytolytic agent $Cl_2MDP$ will be released in the cells, and the cells will be killed (Fig. 1) (236). Since neutrophils are phagocytic cells and are important in host resistance in this disease, it was necessary to determine if L-$Cl_2MDP$ affects neutrophils along with macrophages. In this work, I addressed this issue.

As has been mentioned above, T cells and NK cells produce IFN-γ, which activates macrophages and enhances macrophage and neutrophil candidacidal activity in vitro. In vivo studies gave equivocal results for the role of IFN-γ in host resistance to *Candida* infection (42,103,123,146,304). In these studies, animals were either given IFN-γ or neutralizing antibodies against IFN-γ. Different experimental designs, such as, concentrations of IFN-γ or antibodies, and different routes for giving IFN-γ or antibody to animals may be part of the reasons for the inconclusive data.

Mice with a targeted disruption of the IFN-γ gene (GKO) have been described (67). In the present study, the susceptibility of GKO mice to disseminated candidiasis was examined with the aim of determining whether IFN-γ is essential in host defense against disseminated candidiasis.

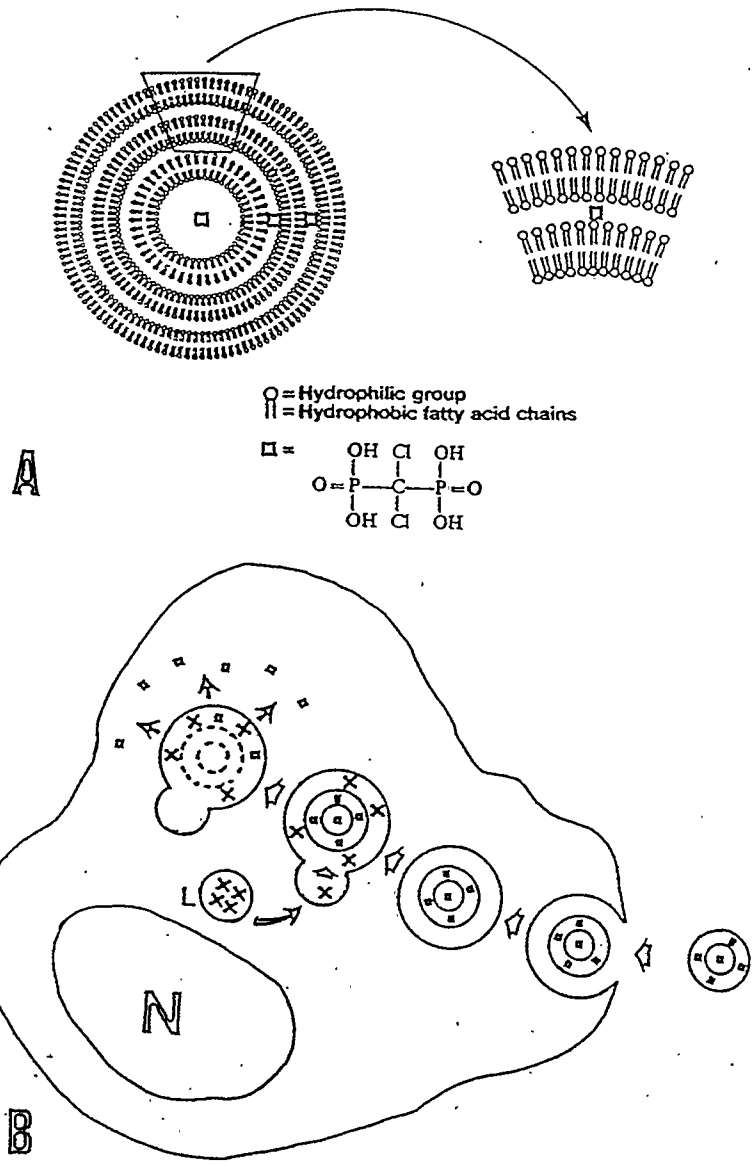


Figure 1. Mechanism of macrophage depletion by L-$Cl_2$MDP (282).

## Chapter 2

# ELIMINATION OF MOUSE SPLENIC MACROPHAGES CORRELATES WITH INCREASED SUSCEPTIBILITY TO EXPERIMENTAL DISSEMINATED CANDIDIASIS

## Introduction

*Candida albicans* is commonly a member of the normal flora of humans, but in 11-27% of neutropenic patients the fungus causes disseminated candidiasis (160), and about 95% of these individuals will die (172). *C. albicans* is a leading cause of disease in patients treated with high doses of broad-spectrum antibiotics and cytotoxic chemotherapies, and in individuals with certain kinds of infectious diseases, such as AIDS (149,269,300).

The increasing clinical importance of *C. albicans*, and diagnostic, treatment and prevention enigmas associated with disseminated candidiasis are reasons to gain an understanding of the interaction between this microorganism and host defense systems. A role for cell-mediated immunity (CMI) in preventing chronic mucocutaneous candidiasis has been documented (38,279). However, the importance of T cells in the immune response against disseminated candidiasis is still a subject of disagreement (38,41,61,151,254) and innate nonspecific immune mechanisms appear to be most important (41,61,187,232).

Of the innate defenses, a considerable number of studies have been done on neutrophils and macrophages. Observations of neutrophils in vitro (51,175), of animals with experimental disseminated candidiasis (9,230), and of patients who develop disseminated

candidiasis (292,300) support the concept that neutrophils are critical in host innate defense mechanisms against this disease.

The importance of macrophages, however, in controlling disseminated candidiasis remains equivocal. Murine peritoneal macrophages (35,64,191,222), pulmonary alveolar macrophages (244), Kupffer cells (245,246) and human peripheral blood monocytes (120,173) show the ability to ingest and kill *C. albicans* in vitro. In addition, murine and human activated macrophages have an increased ability to ingest and kill *C. albicans* in vitro (35,113,295).

In vivo studies on disseminated candidiasis have generated evidence that both supports and denies an importance of macrophages. In supportive studies, nonspecific activation of macrophages by either Bacillus Calmette-Guerin (BCG) or by a poorly virulent agerminative strain of *C. albicans* (strain PCA-2) enhanced the resistance of mice to disseminated disease (8,20). Transfer of splenic macrophages from *Candida*-immunized mice to naive mice enhanced their resistance to the disease (20). Finally, in vivo administration of the macrophage activation factor IFN-γ caused increased protection of mice against disseminated candidiasis (222). In non-supportive reports, in vivo administration of BCG did not increase mouse resistance to the disseminated disease (230) and in vivo administration of murine recombinant IFN-γ either increased their susceptibility (103), or the protective effect of IFN-γ was attributed to neutrophil activation (146). Furthermore, intravenous administration of the macrophage toxin silica into normal BALB/c mice increased their resistance to the disease (152), but this effect did not occur in nude mice

(152), beige mice or their normal littermates (8). More recently, it was even suggested that macrophages play a major role in the pathology of disseminated candidiasis (118).

The importance of macrophages in vivo in host resistance to disseminated candidiasis is controversial in part because it is difficult to specifically eliminate these cells from the host. In addition, in those studies attempting to address the role of macrophages, a consideration of neutrophil functions is usually lacking. In this paper, a recently developed method for in vivo specific elimination of macrophages from the spleen and liver of mice was used (281,283). An increase in susceptibility to disseminated candidiasis was found in macrophage-depleted mice as compared with macrophage-sufficient animals, even when neutrophils in the macrophage-depleted mice appeared normal.

## Materials and Methods

Microorganisms and Culture Conditions. *Candida albicans* strains Ca-1 (Strain 1) (164) and A9 (114) were stored as yeast cells in 50% glycerol at -20 °C and hydrophilic yeast cells were prepared for use as previously described (114).

Mice. Six to eight weeks old BALB/cByJ male and female mice, B6CByf1 congenitally thymic deficient nude (*nu/nu*) female mice and their normal heterozygote female control mice (Jackson Laboratories, Bar Harbor, ME), and IRCf1 male and female mice (Simonsen Laboratories, Gilroy, CA) were used in the experiments.

Liposome Preparation. Multilamellar liposomes were prepared as previously described (265) containing either the cytolytic agent, dichloromethylene diphosphonate ($Cl_2MDP$; a gift from Boehringer Mannheim GmbH, Mannheim, Germany) or phosphate buffered saline (PBS). Briefly, 75 mg phosphatidylcholine and 11 mg cholesterol (Sigma Chemical Co., St. Louis, MO) were dissolved in chloroform, evaporated by rotation under vacuum at 37 °C, dispersed by mixing with 10 ml PBS (0.15 M NaCl; 10 mM phosphate buffer, pH 7.4) containing 2.5 g of $Cl_2MDP$ for 10 min, placed at room temperature (RT, 21-23 °C) for 2 h, sonified for 3 min. at RT in a water bath sonicator and placed at RT for 2 h. The resulting liposomes (L-$Cl_2MDP$) were washed twice in PBS by centrifugation at 100,000 x g for 30 min. to remove untrapped $Cl_2MDP$. The L-$Cl_2MDP$ was suspended in 4 ml PBS, and 0.2 ml of the preparation was given intravenously (i.v.) to mice. The procedure for making PBS-containing liposomes (L-PBS) was the same except that the film of phosphatidylcholine-cholesterol mixture was dispersed in 10 ml PBS without $Cl_2MDP$, and 0.2 ml was given i.v. to mice as a negative control.

Monoclonal Antibodies (mAbs). The following mAbs were used: M1/70, a rat IgG2b anti-Mac-1 α chain (2,260); SK105 and SK208, rat IgG2a antibodies specific for an activation antigen found only on mouse neutrophils [ (131), Jutila unpublished observations]; MONTS-4, a rat IgG2a antibody specific for mouse macrophages found in the splenic marginal zone, corona, germinal center, and cells scattered throughout the periarticular lymphatic sheaths (PALS) of the spleen (130); SK39, a rat IgG2a anti-mouse specific for splenic macrophages found in the red pulp (130); Mel-14, a rat IgG2a antibody specific for

mouse neutrophil homing receptor (L-selectin) (101); and 30G12, a rat IgG2a anti-mouse T200 common leukocyte antigen (150) (a kind gift from Eugene C. Butcher, Stanford University). Each mAb was used as tissue culture supernatant fluid. Appropriate dilutions of the monoclonal antibody preparations were determined by reactivity of each monoclonal culture supernatant with either normal cells for FACS analysis or normal tissue for immunoperoxidase staining.

Immunoperoxidase Staining. Mice were given i.v. 0.2 ml of L-$Cl_2$MDP, L-PBS, or PBS alone, 3 mice per group. Either 3 days or 56 days later, these mice were given i.v. 0.1 ml of 2% luconyl blue (44E 3172; BASF Aktiengesellschaft, Germany), and 5 min. later the mice were sacrificed. The spleens were immediately removed, placed in Tissue Tek O.C.T. compound (Miles Inc., Elkhart, IN), rapidly frozen on dry ice, and stored at -80 °C until use. Spleens were cryosectioned at 5 μm thickness at -20 °C (Frigocut 2800N cryostat; Reichert-Jung, Leica Inc., Deerfield, Ill) and mounted onto glass slides. The sections were air dried, fixed in acetone for 10 min. at 4 °C, air dried again and stored at -80 °C until use. The immunoperoxidase staining was done as previously described (111). Briefly, cryosections were taken from -80 °C, set at RT for 20 min, and overlaid with 100 μl of mAbs SK208, SK39, or MONTS-4. Control tissue sections were overlaid with medium (RPMI 1640 plus 10% FBS) alone. All tissues were incubated for 30 min. at RT, and washed with PBS. The sections were overlaid with 100 μl of biotinylated goat anti-rat immunoglobulin $F(ab')_2$ (TAGO Inc., Burlingame, CA) diluted 1:250 in PBS, incubated for 30 min. as above, washed with PBS, overlaid with 100 μl of streptavidin peroxidase conjugate (TAGO) diluted 1:500

in PBS and incubated as above for 20 min. The sections were washed with PBS, overlaid with 100 µl of the substrate solution, incubated as above for 10 min, washed with tap water and coverslip-mounted with GEL/MOUNT™ (Biomeda Co., Foster City, CA).

Immunofluorescence Staining, FACS Analysis and White Blood Cell Counts. Mice were given i.v. 0.2 ml of L-$Cl_2$MDP, L-PBS or PBS alone. Thirty six hours later, peripheral blood was obtained from the tail artery of each mouse for total white blood cell (WBC) and differential counts, and each mouse was given 1 ml of thioglycollate (BBL Microbiology Systems, Becton Dickinson and Co. Cockeysville, MD) intraperitoneally (i.p.). Four hours later, the mice were sacrificed, and the peritoneal cavity of each was lavaged with 10 ml of Hanks' balanced salts solution without $NaHCO_3$, $Ca^{++}$, and $Mg^{++}$ (GIBCO, Grand Island, NY.) containing 0.05M EDTA (HBSS). Immunofluorescence staining of cells was done in 4 ml glass tubes as previously described (131). The peritoneal cells were pelleted by centrifugation, suspended in 1 ml of HBSS, and 100 µl of each cell suspension was mixed with 100 µl of mAbs Mel-14, M1/70, SK105, or 30G12, incubated for 30 min. on ice, and washed one time with PBS. One hundred microliters of FITC-conjugated $F(ab')_2$ goat anti-rat Ig (TAGO) at a 1/80 dilution in 2% horse serum (HS) (Sigma) in PBS were added to each tube, mixed, incubated for 30 min. on ice, the cells were washed one time with PBS containing 2% HS, and suspended to 0.5 ml in PBS with 2% HS. Fluorescence of the cells was determined by use of a FACScan (Becton Dickinson) and data were collected from 1-5x$10^4$ cells and are presented as mode fluorescence. For peripheral blood neutrophil immunofluorescence staining, blood was obtained from the heart of mice 36 h after giving

i.v. L-$Cl_2$MDP, L-PBS or PBS alone. Sodium citrate (Becton Dickinson Vacutainer System, Rutherford, NJ) was used to prevent blood coagulation. Red blood cells were lysed by treatment with deionized $H_2O$, the remaining cells were washed with HBSS and suspended in HBSS to 0.3 ml, and immunofluorescent stained with mAbs as above.

Neutrophil Phagocytosis of *C. albicans*. An in vitro phagocytosis assay was performed as previously described (191) with minor modifications. In brief, mice were given i.v. 0.2 ml of L-$Cl_2$MDP, L-PBS or PBS alone. Three days later, neutrophil-rich peritoneal cells were obtained from these mice injected i.p. with 2.5 ml of 0.5% glycogen (Sigma Chemical Co.) in saline 3-5 h prior to cell harvest. The peritoneal cells were washed twice with HBSS, once with Medium 199 (M199, GIBCO) and suspended to $4x10^6$ cells/ml in M199. Viability of the peritoneal cells exceeded 90%, and over 80% were neutrophils. One-quarter ml of the peritoneal cell suspension ($4x10^6$ cells/ml in M199 with 5% fresh mouse serum) and 0.25 ml of a suspension of formalin killed *C. albicans* (strain Ca-1) containing $8x10^6$ yeast cells/ml in M199 with 5% fresh mouse serum were added to the surface of cover glasses and incubated at 37 °C for 1 h. The cover glasses were washed with HBSS, air dried, fixed with methanol for 2 min, stained with Giemsa to distinguish neutrophils from other cell types, and examined using light microscopy. All tests were done in duplicate, and 200 cells per cover glass were counted. The percentage of neutrophils that ingested one or more yeast cells, and the average number of yeasts ingested per neutrophil were determined.

An in vivo phagocytosis assay was performed as follows. Mice were treated with L-$Cl_2$MDP or PBS, 3 days later, the animals were injected i.p. with formalin killed Ca-1 yeast

cells $2x10^8$ per mouse. After 5 h, mice were sacrificed, the peritoneal cells were harvested by washing the peritoneal cavity of each mouse with 5 ml of HBSS, cells sedimented by centrifugation, suspended in approximately 0.1 ml HBSS and cell suspension smears were made on glass slides. The cells were stained with Giemsa as above and the percent of neutrophils that ingested yeast cells was determined.

Candidacidal Activity of Neutrophils. The candidacidal activity was tested as previously described (65) with minor modifications. Mice were treated with L-$Cl_2$MDP or PBS, 3 days later, the animals were injected i.p. with 2.5 ml of 0.5 % glycogen in saline. After 3 h, mice were sacrificed, the peritoneal cells were harvested as above with RPMI 1640 with 25 mM HEPES. The peritoneal cells were washed three times with RPMI 1640 with HEPES and suspended to $1x10^6$/ml, $5x10^5$/ml or $2.5x10^5$/ml in RPMI 1640 with 25 mM HEPES and 10 % heat inactivated normal mouse serum. Viability of the peritoneal cells exceeded 90%, and over 80% were neutrophils. Cells were added 0.2 ml/well to 96 well tissue culture plate (Corning Glass Works, Corning, NY) and incubated at 37 °C for 1 h. The supernatant were discarded, 0.2 ml of $1x10^5$/ml live *C. albicans* (Ca-1) hydrophilic yeast cells in RPMI 1640 with HEPES and 10 % fresh mouse serum were added to the 96 well plate and the plate was incubated at 37 °C for 1 h. The supernatant was discarded, 0.2 ml of corn meal agar (42 °C, DIFCO) was added to each well, the plate was incubated at 37 °C for 1 h, and 0.1 ml of 2 % glutaraldehyde was added to fix the fungus. The germ tubes of *Candida* were counted 4 field/well at 250x by use of a reverse bright-field microscope.

Ex vivo Binding Assay. The binding assay was performed as previously described (228). In brief, spleens frozen in O.C.T. were cryosectioned at 10 μm thickness at -20 °C, placed onto glass slides, and air dried. Quadruplicate tissue sections per organ were examined and experiments were done in triplicate for each spleen tissue. One hundred microliters of the *C. albicans* strain A9 (which has identical adherence properties as strain Ca-1) yeast cell suspension were overlaid onto each section and incubated for 15 min. at 4 °C, fixed in 1.5% glutaraldehyde (Fisher Scientific Co., Fair Lawn, NJ) for 16-18 h at 4 °C, washed in cold water, stained with 1% crystal violet and mounted with Permount (Fisher Scientific Co.). Yeast cells that adhered to the spleen marginal zone were counted by computer image analysis as previously described (228).

Determination of the Recovery of *C. albicans* CFU from the Blood of Mice. Mice were given i.v. 0.2 ml of L-$Cl_2$MDP or PBS. Thirty-six hours later, the mice were given i.v. 0.1 ml of *C. albicans* strain Ca-1 yeast cells in PBS containing $1x10^6$ yeasts. After 30 min, 1.5 h, 3 h and 24 h, 0.1 ml of blood from each of three mice per time period was obtained from the tail artery and mixed with 0.3 ml of sterile deionized water. One hundred microliters were plated onto glucose-yeast extract-peptone (GYEP) agar plates, incubated for 48 h at 37 °C and CFU were determined.

Determination of the Recovery of *C. albicans* CFU in Liver, Spleen and Kidneys of Mice. Mice were given i.v. 0.2 ml of L-$Cl_2$MDP or PBS alone. Three days later, each mouse was given i.v. 0.1 ml containing $2.5x10^5$ *C. albicans* strain Ca-1 yeast cells in PBS.

After 1 h, 24 h, 48 h and 72 h, three mice of each treatment at each time period were sacrificed, the liver, spleen and both kidneys from each mouse were removed and weighed. Each liver was homogenized in 5 ml of sterile saline; both kidneys from each animal were homogenized in 5 ml of sterile saline; and each spleen was homogenized in 2 ml of sterile saline. Appropriate dilutions in saline of each homogenate were made and plated onto GYEP agar plates containing 100 u/ml penicillin and 100 µg/ml streptomycin (Microbiological Associates Co. Walkersville, MD), incubated for 48 h at 37 °C and CFU per gram of tissue were determined.

Survival of Mice with Experimental Disseminated Candidiasis. BALB/cByJ, IRCf1, and nude and their normal controls were given i.v. 0.2 ml of L-$Cl_2$MDP, L-PBS, or PBS alone at 5 mice per group. Three days or fifty-six days later, the mice were given i.v. 0.1 ml containing $5x10^5$ or $2.5x10^5$ *C. albicans* strain Ca-1 yeast cells in PBS. The mice were observed daily for survivors for 25 days. In addition, 3 control mice per group were given L-$Cl_2$MDP, L-PBS, or PBS alone and their spleens were removed at day 3 or day 56 for ex vivo binding assays and immunoperoxidase staining as described above.

Statistical Analysis. Differences in survival times were analyzed by the Mann-Whitney test. The student-*t* test was used as a measure of statistical significance in all other data analysis.

# Results

Effect of L-$Cl_2$MDP on Immunoperoxidase Staining. Spleens from both BALB/cByJ mice (Fig. 2) and IRCf1 mice (not shown) pretreated with L-$Cl_2$MDP 3 days before had practically no reactivity with MONTS-4 in the marginal zone, and only minor staining in the white pulp as compared to either L-PBS (not shown) or PBS treated mice whose marginal zone stained heavily. Likewise, L-$Cl_2$MDP treated mice showed minimal red pulp staining with SK39 as compared to L-PBS (not shown) and PBS controls. Splenic tissues from all mice, however, stained equally with the neutrophil-specific mAb SK208. In all experiments, the staining characteristics of mice treated with L-PBS were essentially identical to PBS-treated control animals. The spleens of mice treated with L-$Cl_2$MDP 56 days before sacrificing had almost identical staining characteristics as L-PBS and PBS control mice (not shown). My results confirmed previous observations of macrophage depletion (111,284), and I showed for the first time that neutrophils are not affected by L-$Cl_2$MDP treatment.

Effect of L-$Cl_2$MDP on Neutrophil Activation and Peripheral Blood Counts. After L-$Cl_2$MDP treatment, BALB/cByJ female mouse circulating neutrophils responded normally to thioglycollate. That is, these cells migrated in normal numbers to peritoneum compared to controls. The total peritoneal cell count for L-$Cl_2$MDP, L-PBS or PBS treated mice were $(113\pm38)\times10^4$/ml, $(200\pm72)\times10^4$/ml and $(179\pm32)\times10^4$/ml, respectively, but the differences were not statistically different ($P>0.05$). The peritoneal differential neutrophil counts of L-$Cl_2$MDP treated BALB/cByJ female mice were slightly lower compared to PBS control

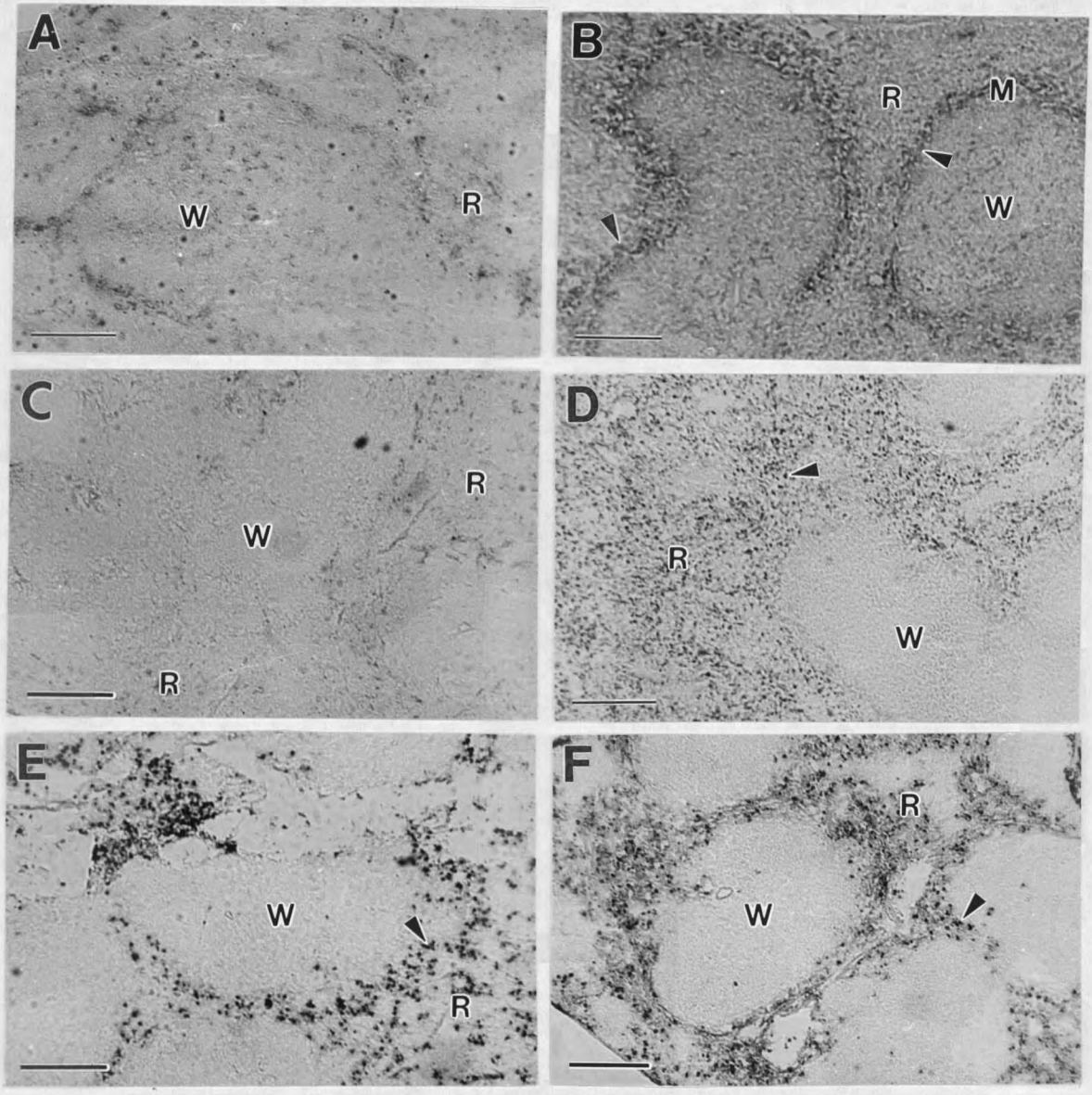


Figure 2. Effect of L-$Cl_2$MDP on immunoperoxidase staining of spleens. BALB/cByJ female mice were given i.v. either L-$Cl_2$MDP or PBS. Three days later, the spleens were removed and immunoperoxidase stained with mAbs MONTS-4 (A and B); SK39 (C and D); and SK208 (E and F). A,C, and E are tissues from L-$Cl_2$MDP treated mice; B, D, and F are tissues from PBS control mice; tissues from L-PBS control mice are not shown. M: marginal zone, R: red pulp, W: white pulp. Bar, 100 μm.

mice, but BALB/cByJ male mice, IRCf1 male and female mice did not show any differences.

Regardless of mouse strain, the percentage of the neutrophils of all mice was about 68±6%.

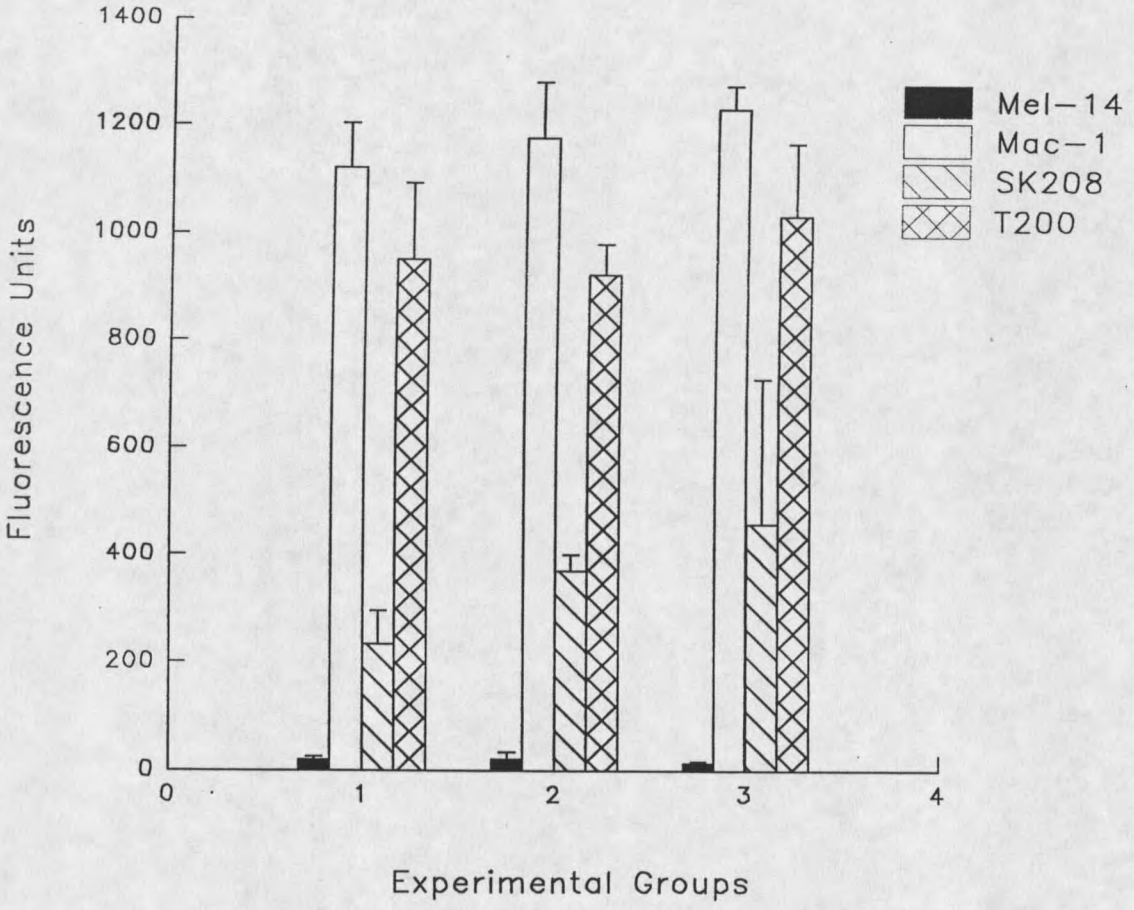


Figure 3. Effect of L-$Cl_2$MDP on neutrophil activation. BALB/cByJ female mice were given i.v. L-$Cl_2$MDP, L-PBS or PBS, and 36 h later, mice were given i.p. thioglycollate. Four hours later, peritoneal cells were obtained, immunofluorescence stained with mAbs Mel-14, Mac-1, SK208, and 30G12, and analyzed by FACScan. The Y axis shows fluorescence units (bars represents standard deviation). Experimental groups 1, 2, and 3 of the x axis represents L-$Cl_2$MDP, L-PBS and PBS treatment, respectively.

Peritoneal elicited neutrophils from L-$Cl_2$MDP treated BALB/cByJ female mice expressed the activation phenotype of high Mac-1 and low Mel-14 antigen levels as did neutrophils from control mice ($P$>0.05) (Fig. 3). Similar results were obtained from BALB/cByJ male and IRCf1 male and female mice (data not shown). These results indirectly showed that

adhesive and migratory functions of blood neutrophils of L-$Cl_2$MDP treated mice were normal.

The total peripheral blood WBC count of L-$Cl_2$MDP treated mice was higher than those of L-PBS or PBS control mice ($P<0.05$), but differential counts were similar in all mice (Table 2). Peripheral blood neutrophils from L-$Cl_2$MDP, L-PBS and PBS treated mice showed approximately 10 times the Mel-14 antigen expression and about one fourth Mac-1 antigen expression as compared to peritoneal exudate neutrophils (data not shown). Monocyte counts remained normal, which indicates that L-$Cl_2$MDP did not affect these cells.

Effect of L-$Cl_2$MDP on Neutrophil Phagocytosis. Glycogen-elicited neutrophils from L-$Cl_2$MDP treated animals showed the same ability to ingest yeast cells as neutrophils elicited from either L-PBS or PBS treated mice in the in vitro phagocytosis assay. The percentages of neutrophils that ingested one or more yeast cells were 78.3±5.3%, 68.2±9.7%, and 67±17.7% for L-$Cl_2$MDP, L-PBS, and PBS treated mice, respectively, and there were no significant differences among these mice ($P>0.05$). Also, there were no differences in the number of yeasts ingested per phagocyte among L-$Cl_2$MDP, L-PBS and PBS treated mice ($P>0.05$), which was about 3 yeasts/phagocyte.

In the in vivo phagocytosis assay, neutrophil phagocytosis activity was not impaired by L-$Cl_2$MDP treatment. The percentage of neutrophils that ingested one or more yeast cells was 12%, 17.7% and 3.5% for three L-$Cl_2$MDP treated mice; and 9.0%, 1.0% and 11.3% for three PBS control mice. There were no differences in the number of yeasts ingested per

Table 2. Effect of L-$Cl_2$MDP on peripheral blood leukocytes.[a]

| Mouse | Treatment | Total WBC ($x10^3/mm^3$) [c] | Blood Cell Differential Count (%) PMN [b] | MC [b] |
|---|---|---|---|---|
| BALB/c female | L-$Cl_2$MDP | 23.5±2.9 | 13±1.5 | 0.7±0.6 |
| | L-PBS | 14.2±2.8 | 18±2.5 | 1±1.7 |
| | PBS | 15.8±1.9 | 10±1.5 | 0.7±0.6 |
| BALB/c male | L-$Cl_2$MDP | 21.3±2.3 | 20±4.2 | 0.3±0.6 |
| | L-PBS | 11.9±2.1 | 19±3.6 | 2±1 |
| | PBS | 15.4±4.4 | 16±5.6 | 0 |
| IRCF1 female | L-$Cl_2$MDP | 21.4±1.7 | 7±2 | 0 |
| | L-PBS | 11.7±1.2 | 13±5.8 | 0.7±0.6 |
| | PBS | 10.6±1.2 | 7.7±2.1 | 0 |
| IRCF1 male | L-$Cl_2$MDP | 16.5±4.1 | 13±3.2 | 0 |
| | L-PBS | 10.6±3.6 | 14±2.5 | 1.3±0.6 |
| | PBS | 12.4±2.4 | 12±3.5 | 1±1 |

[a] Mice were given 0.2 ml of L-$Cl_2$MDP, L-PBS, or PBS, and 36 h later, peripheral blood was obtained from the tail artery. Total WBC and differential counts were made. The total WBC count of L-$Cl_2$MDP treated mice was higher than L-PBS or PBS controls ($P<0.05$). There was no difference in the percentage of neutrophils of L-$Cl_2$MDP treated mice compared to control mice ($P>0.05$). The percentage of lymphocytes was above 76% and there was no difference between L-$Cl_2$MDP treated mice and control mice.

[b] PMN, neutrophils; MC, monocytes.

[c] Mean value and standard deviation of three mice.

phagocyte between L-$Cl_2$MDP and PBS treated mice ($P>0.05$), which averaged 2 yeasts/phagocyte.

Effect of L-$Cl_2$MDP on Neutrophil Candidacidal Activity. The neutrophil candidacidal activity was not impaired by L-$Cl_2$MDP treatment (Table 3). There is no

statistical difference ($P>0.05$) of the killing ability to *C. albicans* between the neutrophils from L-$Cl_2$MDP treated mice and PBS control mice.

Table 3. Effect of L-$Cl_2$MDP on neutrophil candidacidal activity[a]

| | Killing (%) | | |
|---|---|---|---|
| PMN/*Candida* ratio | L-$Cl_2$MDP | PBS | *P* value |
| 10/1 | 78±4 | 83±3 | $P>0.05$ |
| 5/1 | 76±4 | 77±3 | $P>0.05$ |
| 2.5/1 | 68±5 | 66±4 | $P>0.05$ |

[a] BALB/cBy mice were given i.v. L-$Cl_2$MDP or PBS, 3 days later, the animals were given i.p. 0.5 % glycogen. After 3 h, mice were sacrificed, the peritoneal cells were harvested, washed, suspended to different concentrations in RPMI 1640, added to 96 well tissue culture plate and incubated at 37 °C for 1 h. Live *C. albicans* yeast cells in RPMI 1640 with fresh mouse serum were added to the plate and incubated at 37 °C for 1 h. Corn meal agar was added to each well, the plate was incubated at 37 °C for 1 h, and fixed with glutaraldehyde, and germ tubes of *Candida* were counted.

Effect of L-$Cl_2$MDP on Adherence of *C. albicans* to Splenic Tissue. Three days after L-$Cl_2$MDP treatment, mouse splenic tissues had essentially lost the ability to bind yeast cells to the marginal zone (<3 yeasts/field). The spleens from mice treated with L-PBS or PBS showed significantly higher ($P<0.01$) marginal zone yeast cell binding (43-75 yeasts/field) (Fig. 4) than L-$Cl_2$MDP treated mice. In these experiments, L-PBS treated spleens from BALB/cByJ male or female mice and IRCf1 female mice, but not IRCf1 male mice, had lower yeast cell binding ($P<0.05$) than PBS treated control mice (Fig. 4). The reason for the slight reduction in binding in some mice but not others is unclear. By 56 days after L-

$Cl_2MDP$ treatment, the marginal zone binding ability of all mice increased to 45 yeasts/field, which was still slightly less ($P<0.05$) than PBS control mice (55 yeasts/field).

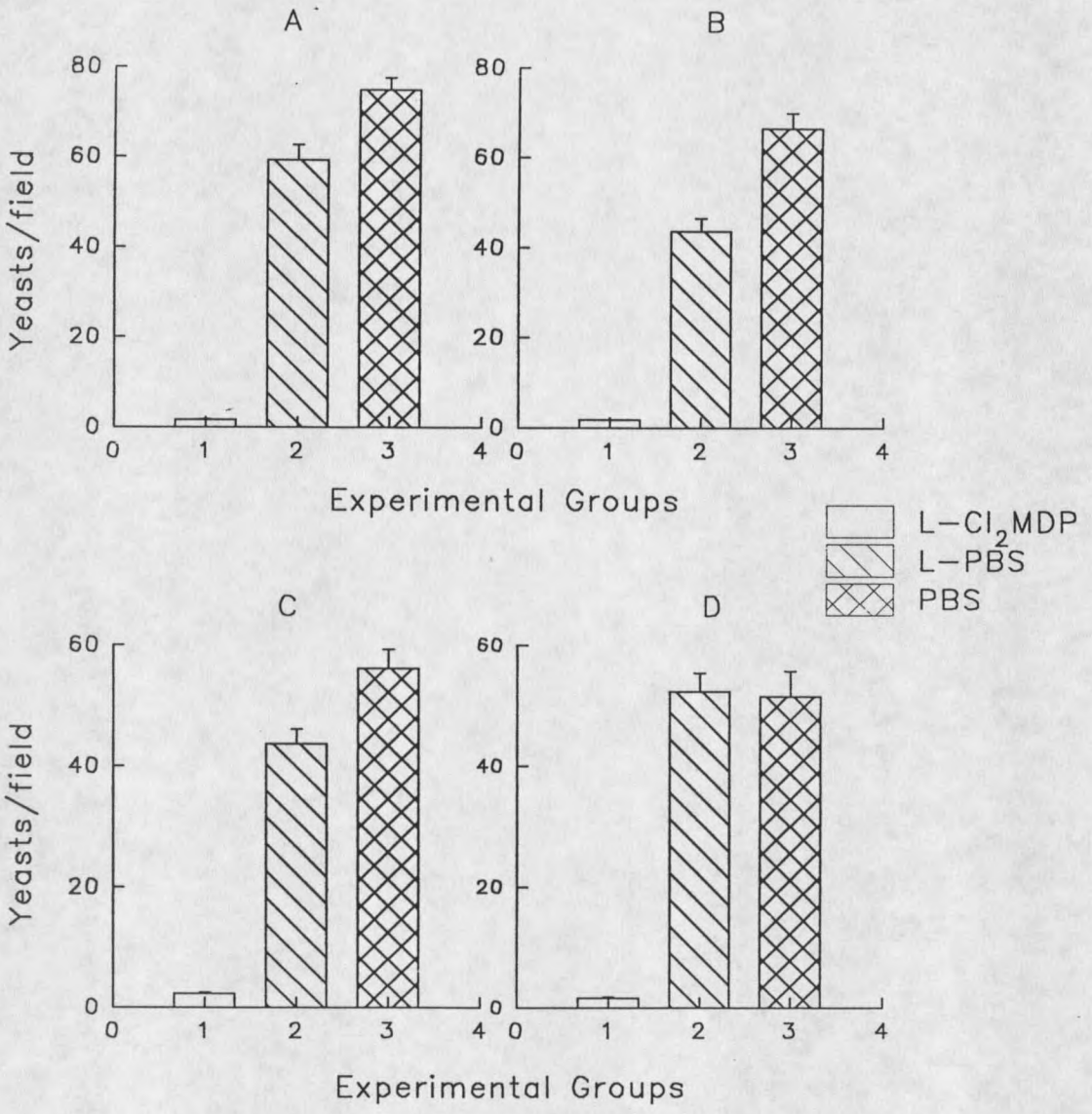


Figure 4. Effect of L-$Cl_2MDP$ on adherence of *C. albicans* yeast cells to splenic tissue. Three days after L-$Cl_2MDP$, L-PBS or PBS treatment, spleens from BALB/cByJ female and male (A and B, respectively) and IRCf1 female and male (C and D, respectively) mice were removed. Ex vivo binding assays were done on cryosections of these spleens. The y axis shows the average number of yeast cells per field that bound to splenic marginal zones (bars represent standard deviation).

Effect of Macrophage Depletion on Clearance of *C. albicans* from Blood. L-$Cl_2$MDP treated mice had, at all time periods tested, a reduced ability to clear yeast cells from their blood (Table 4). At 0.5 h after *C. albicans* infection of mice, the CFU in the blood of L-$Cl_2$MDP treated mice was 4.4 times higher than in PBS control mice. At 1.5 h after *C. albicans* infection, the CFU in the blood of L-$Cl_2$MDP treated mice was about 10 times higher than in PBS control mice.

Table 4. Effect of macrophage depletion on clearance of *C. albicans* from blood[a].

| Time after *C. albicans* infection | Mouse Treatment | | *P* value[c] |
|---|---|---|---|
| | L-$Cl_2$MDP (CFU/ml)[b] | PBS (CFU/ml) | |
| 0.5 h | 1000±131 | 227±217 | $P<0.01$ |
| 1.5 h | 653±50 | 67±50 | $P<0.01$ |
| 3.0 h | 360±98 | 53±38 | $P<0.01$ |
| 24 h | 13±19 | 0 | $P<0.01$ |

[a] BALB/cByJ female mice were given 0.2 ml of L-$Cl_2$MDP or PBS, 36 h later, mice were given i.v. *C. albicans* yeast cells $1x10^6$/mouse. The blood was obtained from the tail artery at different times after *C. albicans* infection, plated onto GYEP agar plates, incubated at 37 °C for 48 h, and CFU were determined.

[b] Mean value and standard deviation of three mice.

[c] Student-*t* test.

Effect of Macrophage Depletion on the Distribution of C. albicans in Liver, Spleen and Kidneys. Spleens from L-$Cl_2$MDP treated mice had fewer CFU per gram of tissue ($P<0.01$) than control mice at 1 h and 24 h, and livers from L-$Cl_2$MDP treated mice had fewer CFU per gram of tissue ($P<0.05$) than control mice at 24 h after a systemic

presentation of yeast cells. By 48 h, however, the fungal load of spleen and liver tissue was similar in all animals ($P>0.05$). The lower initial counts in the spleen and liver may have caused a greater distribution of *Candida* yeasts to the kidneys. The kidneys of L-$Cl_2$MDP treated mice had many more *C. albicans* CFU per gram of tissue ($P<0.01$) than control mice at 24 h, 48 h, and 72 h after a systemic challenge with the fungus (Table 5).

Table 5. Effect of macrophage depletion on the distribution of *C. albicans* in different organs.[a]

| Time after Ca-1 infection | Liver (x$10^3$/g) | | Spleen (x$10^3$/g) | | Kidney (x$10^3$/g) | |
|---|---|---|---|---|---|---|
| | L-$Cl_2$MDP | PBS | L-$Cl_2$MDP | PBS | L-$Cl_2$MDP | PBS |
| 1 h | 15±0.1 | 37±3 | 10±2 | 30±4 | 30±4 | 22±7 |
| 24 h | 1±1 | 14±3 | 2±2 | 25±2 | 4,567±1,160 | 811±116 |
| 48 h | 3±1 | 3±0.2 | 23±9 | 9±1 | 42,837±6,402 | 2,485±2,143 |
| 72 h | 8±3 | 3±1 | 42±26 | 9±2 | 42,463±5,774 | 391±254 |

[a] BALB/cByJ female mice were given i.v. L-$Cl_2$MDP or PBS. Three days later, each mouse was given i.v. 2.5x$10^5$ *C. albicans* yeasts cells. The organs were removed at different times, homogenized and plated for CFU determinations. Values are mean ± SD from three mice.

Effect of Macrophage Depletion on Experimental Disseminated Candidiasis. Three days after the L-$Cl_2$MDP treatment, BALB/cByJ male and female mice, IRCf1 male mice (Fig. 5), were more susceptible ($P<0.01$) to experimental disseminated candidiasis than control mice, and there was no significant difference between L-PBS and PBS treated mice ($P>0.05$). L-$Cl_2$MDP treated IRCf1 female mice also showed a similar tendency to be more susceptible to disseminated candidiasis, but the results were not significantly different ($P>0.05$) compared to either L-PBS or PBS control animals (Fig. 5). Furthermore, nude

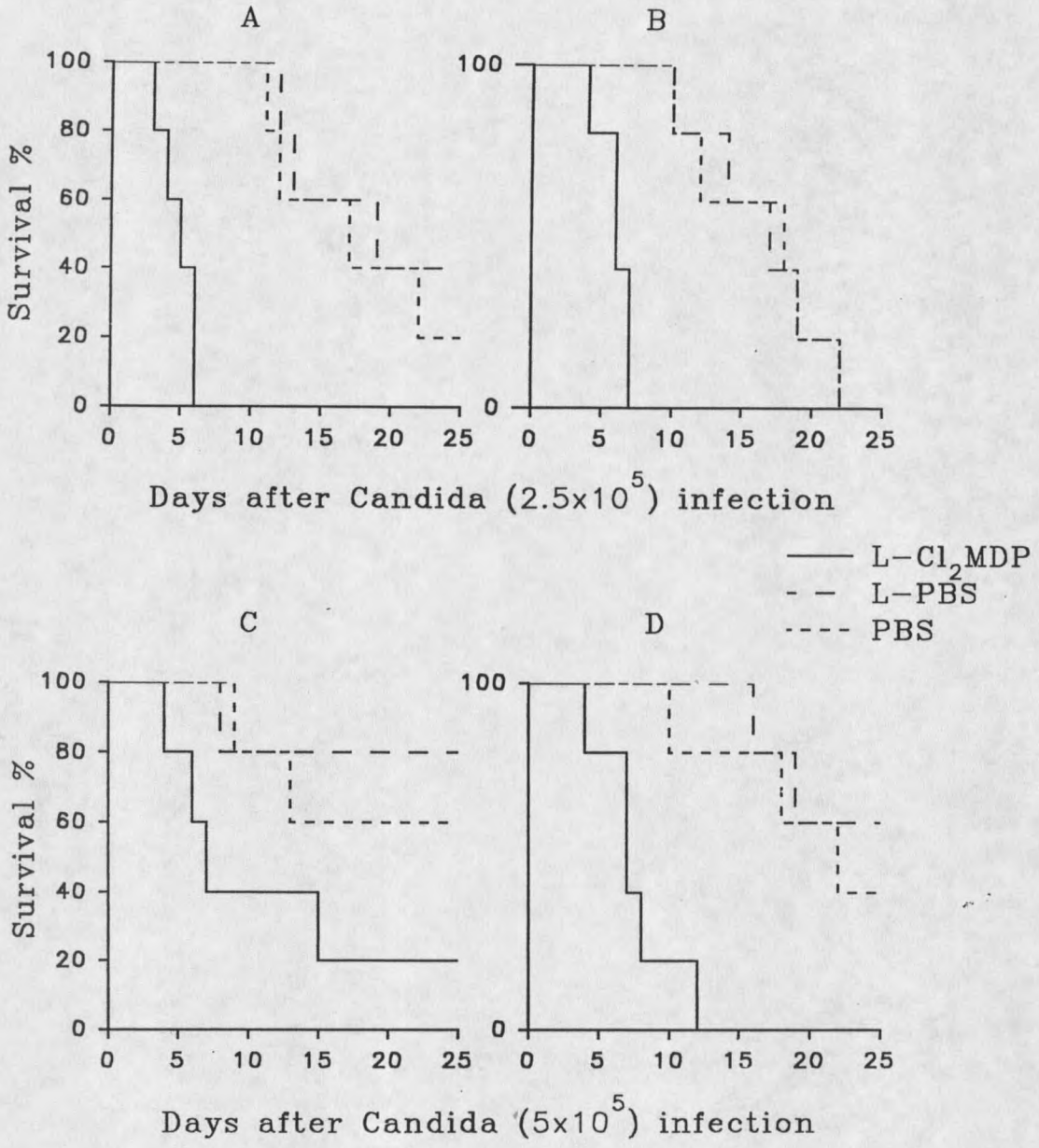


Figure 5. Effect of macrophage depletion on experimental disseminated candidiasis. BALB/cByJ female and male mice (A and B, respectively), and IRCf1 female and male mice (C and D, respectively) were treated with L-$Cl_2$MDP, L-PBS or PBS. Three days later, mice were systemically challenged with *C. albicans* yeast cells, and were observed daily for survival for 25 days. BALB/cByJ female and male, and IRCf1 male mice were more susceptible to experimental disseminated candidiasis ($P<0.01$, Mann-Whitney test).

mice and their normal control mice showed similar results as BALB/cByJ mice. When the mice were treated with L-$Cl_2$MDP or PBS for 3 days and then challenged i.v. with *C albicans* $2.5x10^5$ yeast cells, 80% of L-$Cl_2$MDP treated mice died within 14 days after infection; while 80-100% of mice treated with PBS were still alive on day 25. Mice treated with L-$Cl_2$MDP 56 days before fungal challenge did not show increased susceptibility to disseminated candidiasis ($P>0.05$; data not shown).

## Discussion

Liposome encapsulated dichloromethylene diphosphonate (L-$Cl_2$MDP) was used to selectively deplete mice of macrophage function (284). This reagent depleted macrophages in the spleen as evidenced by immunoperoxidase staining with mAbs against mouse splenic macrophages, and the macrophage specificity of the L-$Cl_2$MDP is similar to our previous finding (111). Furthermore, when mice were treated with L-$Cl_2$MDP, the marginal zone of the spleen lost its ability to bind *C. albicans* yeast cells. This result is in agreement with our report that *C. albicans* yeast cells bind specifically to marginal zone macrophages of mouse spleen (134). Background staining difficulties precluded our ability to analyze the effects of L-$Cl_2$MDP on liver tissues, however, an intravenous delivery of L-$Cl_2$MDP is known to deplete liver Kupffer cells (48,281,283). In accordance with these observations, I found that trapping of *C. albicans* yeast cells by the liver was impaired in animals treated with the L-$Cl_2$MDP preparation (Table 4). This result agrees with others who reported that Kupffer cells are responsible for trapping *C. albicans* yeast cells (245,246).

The L-$Cl_2$MDP did not affect neutrophil number or function in treated mice. The higher total WBC count of L-$Cl_2$MDP treated mice as compared to L-PBS or PBS control mice (Table 2) may be due to bone marrow stimulation in response to macrophage depletion. It is not known if neutrophils are also depleted in mice within 36 h of L-$Cl_2$MDP treatment. Neutrophils in the host may recover to normal within this period, because the turn over time for neutrophil is about 12 hours. After 36 h after L-$Cl_2$MDP treatment, when I challenged mice with *C. albicans*, the neutrophils are normal both in number and function. The ratios of Mac-1 to Mel-14 on neutrophils from the blood and from the inflamed peritoneum corresponded to normally functioning neutrophils (138). Moreover, peritoneal exudate neutrophils of L-$Cl_2$MDP treated mice had the same ability to ingest formalin-killed *C. albicans* yeast cells either in vitro or in vivo and to kill *Candida* yeast cells in vitro as neutrophils from control mice. These data showed that, while macrophages were depleted, the L-$Cl_2$MDP treatment did not appear to affect neutrophils, and $Cl_2$MDP treated mice should be a useful model to probe neutrophil function.

Depletion of macrophages from mouse spleen and, presumably, liver increased the susceptibility of mice to experimental disseminated candidiasis as indicated by several measures. First, macrophage-depleted mice had impaired clearance of *C. albicans* yeast cells from the blood at 1 h following an i.v. presentation of the fungus. Second, in accordance with our findings that splenic macrophages entrap *C. albicans* yeast cells both in ex vivo (133,134) and in vivo (133) experiments, macrophage-depleted mice had impaired ability to entrap yeast cells in the spleen. At 1 h and 24 h after an i.v. inoculum of viable yeast cells,

spleen tissue from L-$Cl_2$MDP-treated mice had lower CFU/g tissue than normal control mice (Table 4). Third, in mice that received an i.v. challenge of yeast cells, kidneys from L-$Cl_2$MDP-treated mice had more recoverable CFU/g tissue than control mice. Counts of fungal units is an important measure because increased CFU in the kidneys have been shown to correlate with severity of disseminated candidiasis in mice (61). Fourth, macrophage-depleted mice did not survive as long as control mice to experimental disseminated candidiasis.

The mechanism by which macrophages serve in protection against disseminated candidiasis is not known. It is possible that macrophages either kill the fungus or secrete cytokines, e.g. IL-1 and TNF-α, to enhance functions of other effector cells. We (61) and others (151) found that congenitally thymic deficient nude mice are more resistant to disseminated candidiasis than their normal littermates. Clinical evidence also shows that patients who are T-cell deficient and develop chronic mucocutaneous candidiasis (199) including AIDS patients (293), rarely develop deep tissue disseminated candidiasis. Further, my results on nude mice in survival experiments suggest that thymus derived T cells are not involved in this macrophage protection mechanism.

My data conflict with previous reports that elimination of macrophages causes an increased resistance of mice to disseminated candidiasis (152). The macrophages in these studies were depleted by an i.v. administration of silica which makes interpretation difficult. The silica-induced resistance did not occur in nude mice (152), or in beige mice and their normal littermates (8). The mechanisms by which silica exerts its effect have not been

elucidated (152,259) and factors such as size and dose of the silica particles may affect the results. In addition, silica may cause macrophages to produce cytokines such as IL-1 (259). In my investigations reported here and the work of others, L-$Cl_2$MDP appears to deplete only macrophage function (47,49).

My results strongly support the idea that macrophages are important in host defense against experimental disseminated candidiasis.

# Chapter 3

## IFN-γ IS NOT ESSENTIAL IN HOST DEFENSE AGAINST DISSEMINATED CANDIDIASIS IN MICE

### Introduction

*Candida albicans* is an increasingly important opportunistic fungal pathogen in immunocompromised patients. It is now the fourth leading cause of nosocomial blood stream infections (122). Hematogenous disseminated candidiasis has increased dramatically as a result of wide-spread use of chemotherapeutic drugs, indwelling catheters and certain kinds of surgical and other medical procedures (73). This serious disease often leads to death even with treatment (172). Reasons for treatment failures are complex and may include problems with fungal resistance and/or toxicity of these drugs to the host (106,221). Clarification of host defense mechanisms against *C. albicans* may well lead to improved methods of prevention and treatment of this disease through immune therapy.

Components of both innate non-specific immune responses and acquired specific immunity have been shown to be important in host defense against disseminated candidiasis. Studies on neutrophils in vitro (175), in animal models (9), and in patients who develop disseminated disease (292) indicate that neutrophils are a critical innate host defense mechanism against this disease. Results from in vitro studies (113) and from experimental

animals (20,125,216,285) also show that macrophages are important in host defense against candidiasis. For specific immune defense against the disease, antibodies have been shown to protect mice against disseminated candidiasis (109,178,192,267). T cell deficient patients, such as those with AIDS, usually do not develop this disease. Furthermore, T cell deficient mice, such as *scid* (14) and nude mice (61,151) are not more susceptible to acute phase disseminated candidiasis. Studies of in vitro and in animal models, however, show evidence that T cells may protect the host against this disease. For example, $CD8^+$ T cells can inhibit the growth of *C. albicans* hyphae in vitro (18), and a *C. albicans* specific T cell line (254) and Th 1 predominant immune responses (234) protect mice against disseminated candidiasis.

IFN-γ, which is mostly produced by T cells and NK cells (274), induces activation of macrophages and enhances both macrophage and neutrophil anti-*Candida* activity in vitro. For example, IFN-γ augments intracellular killing of *Candida* yeast cells by macrophages (174) and neutrophils (262), and hyphal killing by neutrophils (77). While these investigations suggest a role for IFN-γ in host defense against disseminated candidiasis, in vivo studies are inconclusive. Administration of an interferon stimulator, poly (I:C), increased the severity of experimental candidiasis (123,304). Treatment of mice with neutralizing anti-IFN-γ monoclonal antibodies caused increased susceptibility to disseminated disease in one study (42), but others found that this treatment resulted in increased resistance (123). In another investigation, administration of recombinant IFN-γ protected mice against the disease (146), whereas others showed that mice who received the

cytokine had increased susceptibility to this form of candidiasis (103).

Mice with a targeted disruption of the IFN-γ gene have been described (67). These IFN-γ gene knock-out (GKO) mice appear normal, fertile, and healthy in the absence of pathogens. Furthermore, they appear not to have alterations in splenic or thymic cell populations (67). In the present study, I examined the susceptibility of GKO mice to disseminated candidiasis with the aim of determining whether IFN-γ is essential in host defense against this disease.

## Materials and Methods

Microorganisms and Culture Conditions. *C. albicans* strains Ca-1 (strain 1, serotype A) (33,162,163) and Ca-222 (serotype B) (63) were described previously. They were stored as yeast cells in 50% glycerol at -20 °C, and were prepared as washed hydrophilic stationary phase yeast form cells in sterile Dulbecco's phosphate buffered saline (DPBS) as previously described (114). Strain Ca-1 was used in all studies unless indicated otherwise.

Mice. Mating pairs of heterozygote BALB/c mice were kindly provided by Genentech, Inc. (San Francisco, CA), and were bred for GKO (homozygous recessive, g/g), wild-type (WT) (homozygous normal dominant, w/w), and heterozygous (w/g) mice in an AALAC-certified Animal Resource Center at our university. The genetic designations are the same as those used in the original description of these mice (67).

PCR Genotyping of Mice. F1 offsprings of the original heterozygote mating pairs were screened for GKO and WT genotypes by PCR amplification of DNA primed specifically for either the normal (5'-AGAAGTAAGTGGAAGGGCCCAGAAG-3' and 5'-AGGGAAACTGGGAGAGGAGAAATAT-3') or the disrupted (5'-TCAGCGCAGGGGCGCCCGTTCTTT-3' and 5'-ATCGACAAGACCGGCTTCCATCCGA-3') IFN-γ gene. The specific primers were obtained from Microcellular Resources, Fort Collins, CO (67). Bacteriophage λ DNA specific primers (5'-GATGAGTTCGTGTCCGTACAACTGG-3' and 5'-GGTTATCGAAATCAGCCACAGCGCC-3') (PCR control) and other reagents used for PCR were from GeneAmp® PCR Reagent Kit with AmpliTaq® DNA Polymerase (Perkin Elmer, Norwalk, CT). Briefly, about 4 mm of mouse tail was cut from each animal, digested in 0.04 % proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) containing 5% SDS, 0.1 M NaCl, 50 mM Tris-HCl and 7.5 mM EDTA, pH 7.2 at 60 °C for 16 h, and DNA was purified by chloroform extraction and precipitation by ethanol. DNA preparations were suspended in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and stored at 4 °C until use. The DNA samples were amplified in the presence of the specific primers (PTC-100 Programmable Thermal Controller, MJ Research, Inc., Watertown, MA). The amplified DNA was electrophoretically run on a 1.7 % agarose gel, and stained with ethidium bromide.

Phenotype of Mice as Determined by ELISA. In addition to genetic confirmation, I used an IFN-γ-specific ELISA (PharMingen, San Diego, CA) to measure IFN-γ protein produced in the supernatant fluid of mouse splenocytes stimulated by concanavalin A (Con

A, Sigma Chemical Co. St. Louis, MO). In brief, spleens were obtained from 6- to 8-week-old mice. A syringe and a needle were used to gently flush each spleen with 8 ml of RPMI 1640 complete medium (CM, Sigma Chemical Co.), which contained 10% fetal bovine serum, 20 mM HEPES (Sigma Chemical Co.), 50 U/ml penicillin and 50 μg/ml streptomycin (Microbiological Associates Co., Walkersville, MD) and 2 mM L-glutamine (Sigma Chemical Co.). The red blood cells in the resulting splenocyte preparation were lysed by treatment with distilled water for 10 seconds at room temperature, and the remaining cells were washed with CM and suspended to $5x10^6$ or $1x\ 10^7$ cells/ml of CM containing 5 μg/ml Con A. The suspension of splenocytes was incubated in wells (1 ml/well) of a 24 well polystyrene plate (Corning Glass Works, Corning, NY) for 72 h at 37 °C under 5% $CO_2$. The supernatant material was collected, aliquoted, and stored at -80 °C. Aliquots were thawed and tested for IFN-γ by ELISA (96).

Determination of *C. albicans* CFU in Mouse Organs. For mice infected intravenously (i.v.), 6- to 8-week-old GKO and WT mice were given i.v. $5 \times 10^5$ or $2.5 \times 10^5$ *C. albicans* yeast cells in DPBS. Forty-eight or 72 h later, mice were sacrificed by cervical dislocation, and liver, spleen and kidneys from each mouse were removed and weighed. Each liver or both kidneys were homogenized in 5 ml of sterile saline, and each spleen was homogenized in 1.5 ml of sterile saline. Appropriate dilutions in saline of each homogenate were made and plated onto glucose (2 %)-yeast extract (0.3 %)-peptone (1%) (GYEP) agar plates containing 100 U/ml penicillin and 100 μg/ml streptomycin (Microbiological Associates Co.), incubated for 48 h at 37 °C, and *Candida* CFU per gram of tissue were

determined.

Five- to six-day-old infant mice were used for intragastric (i.g.) inoculations with *C. albicans*. The GKO or WT infant mice were isolated from their mothers and kept at 35 °C for 3 h before i.g. inoculation with 2 x $10^8$ or 5 x $10^8$ *C. albicans* strains Ca-1 or Ca-222 yeast cells in 50 µl DPBS. The mice were returned to their respective mothers after inoculation. Quantitative cultures of lung or gastric contents performed immediately after the i.g. inoculation showed that the entire inoculum reached the stomach. Three hours, 10 days and 21 days after i.g. inoculation, the mice were sacrificed by decapitation, and lung, liver, spleen, kidneys, stomach and intestines were removed and weighed. Before the mice were sacrificed, they were isolated from their mothers for 1-2 h to allow their stomachs to empty. Each lung, liver, spleen or both kidneys were homogenized in 2 ml of sterile saline, and each stomach or whole intestine was homogenized in 5 ml of sterile saline. Appropriate dilutions in saline of each homogenate were made and plated onto Mycobiotic agar plates (DIFCO Laboratories, Detroit, MI). The plates were incubated for 72 h at 37 °C, and CFU per gram of tissue were determined.

Survival of mice with Experimental Disseminated Candidiasis. Six- to eight-week-old GKO and WT control mice at 5 mice per group were given i.v. 0.1 ml containing 1 x $10^6$, 5 x $10^5$, or 2.5 x $10^5$ *C. albicans* yeast cells in DPBS. The mice were observed daily for survivors for up to 40 days.

Histological Studies of Stomach and Jejunum of Mice Infected Intragastrically with *C. albicans*. Five- to six-day-old infant GKO or WT mice were given i.g. 2 x $10^8$ *C. albicans* yeast cells. Twenty-one days later, mice were sacrificed, and their stomach and jejunum were removed. For each mouse, the stomach cardial-atrium-fold (CAF) regions were cut into 2 pieces and 4 pieces from the middle part of the jejunum was collected and fixed in 10% formalin. The 2 pieces of stomach were embedded in one paraffin wax block and the 4 pieces of jejunum were embedded in one paraffin wax block. The tissues were cut at 5 μm thick sections at five different levels, five sections of each level were stained with Grocott methenamine silver (GMS) and five sections were stained with hematoxylin and eosin (H & E). The tissues were evaluated by the following criteria: (1) observe for evidence of hyphal and yeast invasion beyond the keratinized layer, and for hyphal extension into the epithelium; (2) make these observations at the five different levels of the stomach from each mouse; (3) observe for fungal load in the tissue between the two groups of mice; and (4) observe the extent of infiltration of inflammatory cells associated with the presence of fungi.

In vivo Induction of MHC Class II IA Antigen Expression on Peritoneal Macrophages. As a preliminary to these experiments, about 93% of normal BALB/c mouse peritoneal macrophages were shown to up-regulate IA expression following stimulation with IFN-γ as detected by the following methods. GKO and WT mice were given intraperitoneally (i.p.) 5 x $10^5$ *C. albicans* yeast cells in 2 ml DPBS or DPBS control. Four days later, mice were sacrificed, peritoneal cells were obtained by washing the peritoneal cavity with RPMI 1640 complete medium (CM) as above. The cells were washed three

times with CM, suspended in CM to a concentration of 2 x $10^5$ cells/ml, and incubated at 1 ml/well in 4-well tissue culture chamber slides (Nalge Nunc International, Naperville, IL) at 37 °C, 5% $CO_2$ for 2 hr. Adherent cells (macrophages) were obtained by washing the wells twice vigorously at room temperature with Hank's balanced salts solution (GIBCO laboratories, Grand Island NY), air dried, fixed in acetone at 4 °C for 7 min., and stored at -80 °C. The cells were stained with biotin-conjugated mouse anti-mouse $IA^{-d}$ monoclonal antibody (PharMingen, San Diego, CA) and immunoperoxidase as described previously (216). Percentages of IA positive cells were obtained by counting a total of 200 cells per sample by use of a bright-field microscope.

Statistical Analysis. The Mann-Whitney test was used for comparisons of colony forming units from cultures of tissues and for analysis of survival experiments. The Student-*t* test was used as a measure of significance for comparing levels of IFN-γ produced by stimulated splenocytes from the various kinds of animals and for IA antigen expression analysis.

## Results

Testing and Confirmation of Genotype of GKO Mice. Genotypes of offspring mice from heterozygote mouse mating pairs were tested to breed homozygote GKO and WT mice. As shown in Fig. 6, the PCR amplified WT IFN-γ genomic band (w) was about 230 base pairs (bps) and the GKO band (g) containing the IFN-γ disruption (neomycin band) was about 373 bps. The bacteriophage λ DNA PCR control band (λ) was about 518 bps. The

DNA samples showing only a normal IFN-γ band were from WT mice (w/w), those with only a neomycin band were from homozygote GKO mice (g/g), and those showing both bands were from heterozygote mice (g/w).




Figure 6. PCR confirmation of genotypes of GKO, heterozygote and WT control mice. WT IFN-γ genomic band(w) is about 230 base pairs (bp), GKO disrupted IFN-γ genomic band (g) is about 373 bps; and bacteriophage λ DNA band is about 518 bps. Samples from homozygote GKO mice showed only a g band (g/g); heterozygote mice had both g and w bands (g/w); and wild type mice had only a w band (w/w). The control bacteriophage lambda DNA band (λ) is also shown.

GKO mice were also confirmed by the ELISA test for IFN-γ. The splenocytes of the three GKO mice stimulated with Con A did not produce detectable IFN-γ, while the splenocytes of the three WT control mice produced 1.38, 7.37 and 1.58 IFN-γ ng/ml splenocyte culture supernatant, respectively. The minimum detection of this test is 79 pg/ml of IFN-γ. These results indicate that GKO mice do not have a normal IFN-γ gene and do not produce detectable levels of IFN-γ.

Effect of IFN-γ Gene Knock-out on the Distribution of *C. albicans* in Liver, Spleen, and Kidneys of Mice Infected Intravenously. The lack of IFN-γ may result in abnormal phagocytes' functions. This may cause a change in the distribution of the fungus in the

organs rich in macrophages and neutrophils when *Candida* were given i.v. to the mice. Also, a higher load of *Candida* CFU may found in the kidneys. I found no significant difference of *Candida* CFU recovered from livers, spleens or kidneys between GKO and WT mice in the two experiments (Table 6). *P* values were calculated for each set of data and none was less than 0.05. Surprisingly, the CFU of the kidneys of GKO mice tended to be less than that from WT mice.

Table 6. Distribution of *C. albicans* in different organs of GKO and WT mice after intravenous infection[a]

| | | Liver ($x10^3$CFU/g) | | Spleen ($x10^3$CFU/g) | | Kidneys ($x10^3$CFU/g) | |
|---|---|---|---|---|---|---|---|
| | | GKO | WT | GKO | WT | GKO | WT |
| | 1[b] | 0.1 | 0.1 | 1.0 | 1.6 | 4 | 12 |
| Exp.1 | 2 | 0.2 | 0.2 | 1.0 | 0.4 | 26 | 89 |
| | 3 | 0.3 | ND | 0.2 | 0.3 | 185 | 18 |
| | 1 | 5 | 4 | 17 | 11 | 32,270 | 7,701 |
| | 2 | 18 | 3 | 78 | 17 | 160 | 3,362 |
| Exp.2 | 3 | 8 | 3 | 7 | 11 | 79 | 3,255 |
| | 4 | 24 | 4 | 57 | 30 | 534 | 49,716 |
| | 5 | 6 | 6 | 10 | 20 | 2,480 | 47,504 |

[a] GKO and WT mice were given i.v. $2.5x10^5$ (experiment 1, Exp.1) or $5x10^5$ (Exp.2) viable *C. albicans* yeast cells. Three days (Exp.1) or 48 h (Exp.2) later, various organs were removed, homogenized, and plated for determination of CFU per gram of tissue. Values are from individual mice. There was no difference between GKO and WT mice in each set of data ($P>0.05$).

[b] Individual mice.

Effect of IFN-γ Gene Knock-out on Hematogenous Disseminated Candidiasis of Mice. To see if mice lacking IFN-γ would be more susceptible to disseminated candidiasis, I observed survival of GKO and WT mice to an i.v. challenge with *C. albicans*. I found that GKO mice survived as long as WT mice at different infection doses (Fig. 7A, B, C, D and F). For each set of data, the *P* value is >0.05. Yet in one experiment (Fig.7E), GKO mice survived longer than control mice ($P<0.05$). These results indicated that GKO mice were not more susceptible than WT mice to death by intravenous infection.

Effect of IFN-γ Gene Knock-out on Colonization and Dissemination of *C. albicans* in Mice Infected by the Intragastric route. To test if IFN-γ may be essential to the host in limiting *Candida* spread from mucosal sites (see discussion below), I observed fungal colonization and dissemination of mice which were given *Candida* i.g. Three hours, 10 days and 21 days after i.g. inoculation of *C. albicans*, no dissemination of *Candida* was detectable in the lung, liver, spleen or kidneys of either GKO or WT mice. The CFU of the kidneys of one mouse was due to only one CFU on the plate of undiluted homogenate. There was also no significant difference of the CFU in the stomach or intestines between GKO and WT mice at different time courses (Table 7). For each comparison set of data, the *P* value was >0.05. GKO mice inoculated i.g. with *C. albicans* strain Ca-222, produced essentially identical results 3 h after inoculation (data not shown).

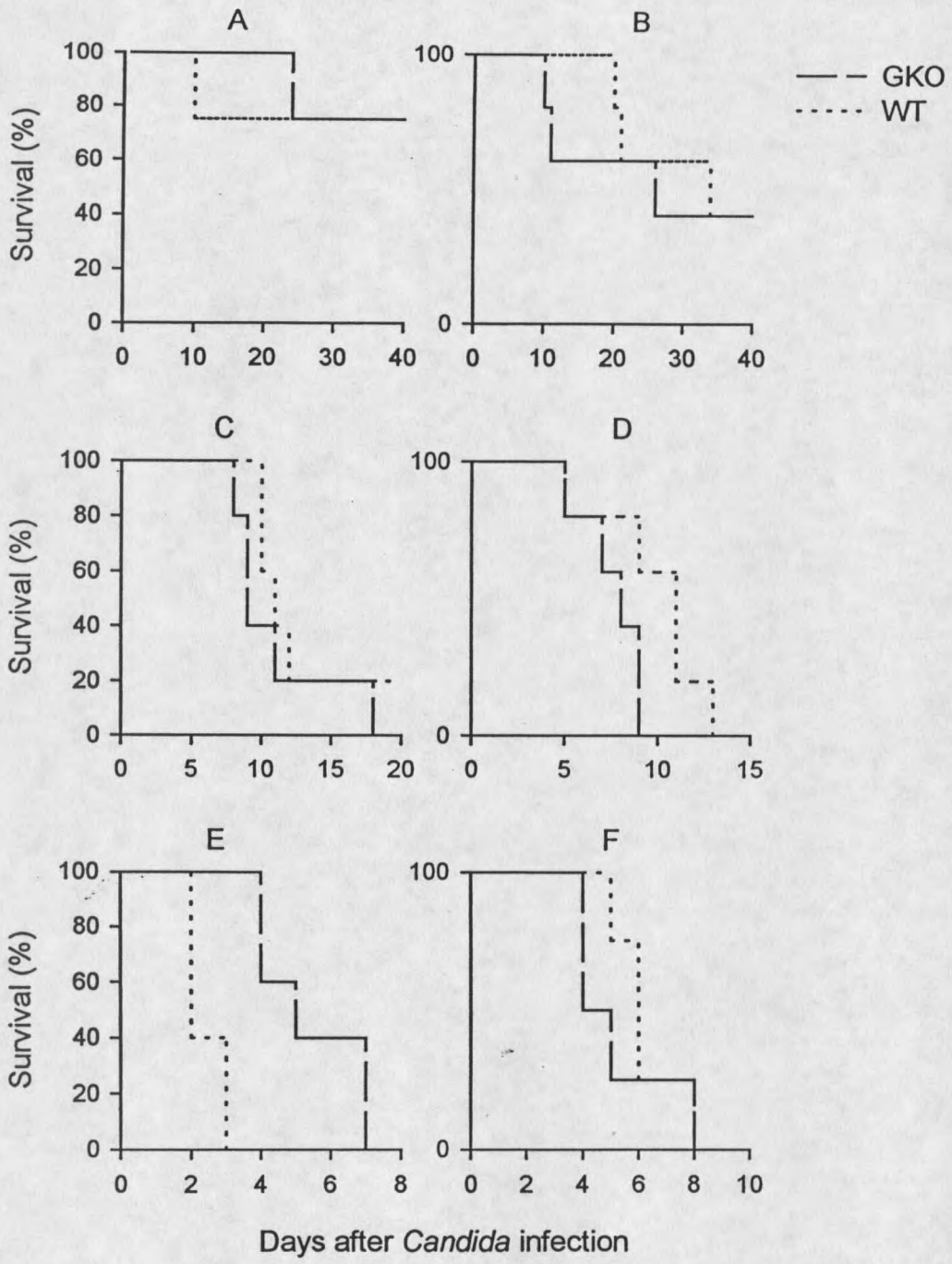


Figure 7. Effect of IFN-γ gene knock-out on hematogenous disseminated candidiasis in mice. GKO and WT mice were infected i.v. with $2.5x10^5$ (A and B), $5x10^5$ (C and D), or $1x10^6$ (E and F) *C. albicans* yeast cells, and were observed for survivors for up to 40 days. The data are from four experiments. There was no significant difference between GKO and WT mice in A, B, C, D, and F ($P>0.05$), but GKO mice survived longer than WT mice in E ($P<0.05$).

Table 7. Effect of IFN-γ gene knock-out on *C. albicans* colonization and dissemination in the GI tract[a]

| Time | Mice | | CFU ($x10^4$)/g tissue | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Lung | Liver | Spleen | Kidneys | Stomach | Intestines |
| | | 1[b] | 0 | 0 | 0 | 0 | 2,216 | 26,557 |
| | GKO | 2 | 0 | 0 | 0 | 0 | 980 | 32,754 |
| | | 3 | 0 | 0 | 0 | 0 | 3,667 | 41,148 |
| 3 h | | | | | | | | |
| | | 4 | 0 | 0 | 0 | 0 | 818 | 35,000 |
| | WT | 5 | 0 | 0 | 0 | 0 | 7,690 | 42,333 |
| | | 6 | 0 | 0 | 0 | 0 | 1,281 | 33,684 |
| | | 1 | 0 | 0 | 0 | 0 | 6 | 2 |
| | | 2 | 0 | 0 | 0 | 0 | 0.4 | 0.4 |
| | GKO | 3 | 0 | 0 | 0 | 0 | 15 | 1 |
| | | 4 | 0 | 0 | 0 | 0 | 6 | 1 |
| 10 d | | 5 | 0 | 0 | 0 | 0 | 5 | 1 |
| | | 6 | 0 | 0 | 0 | 0 | 18 | 2 |
| | WT | 7 | 0 | 0 | 0 | 0 | 12 | 1 |
| | | 8 | 0 | 0 | 0 | 0 | 11 | 2 |
| | | 1 | 0 | 0 | 0 | 0 | 17 | 1 |
| | GKO | 2 | 0 | 0 | 0 | 0 | 81 | 13 |
| | | 3 | 0 | 0 | 0 | 0.016 | 147 | 5 |
| | | 4 | 0 | 0 | 0 | 0 | 23 | 2 |
| 21 d | | | | | | | | |
| | | 5 | 0 | 0 | 0 | 0 | 35 | 2 |
| | WT | 6 | 0 | 0 | 0 | 0 | 26 | 2 |
| | | 7 | 0 | 0 | 0 | 0 | 9 | 1 |
| | | 8 | 0 | 0 | 0 | 0 | 10 | 2 |

[a] GKO and WT mice were given i.g. $2x10^8$ viable *C. albicans* yeast cells. Three hours, 10 d and 21 d later, various organs were removed, homogenized, and plated for determination of CFU per gram of tissue. Values are from individual mice.

[b] Individual mice.

As a control for the neonatal mouse model, splenocytes from infant mice were evaluated for their ability to produce IFN-γ. Splenocytes from 6-, 16-, and 27-day-old mice were compared with splenocytes from 6-8 week old mice (3 mice/group). Following stimulation with Con A, the amount of IFN-γ (ng/ml splenocyte culture supernatant) was 4.0 (±2.2), 6.0 (±0.10), 5.84 (±0.16) for 6-, 16- and 27-day-old mice, respectively, and 5.75 (±0.16) for 6-8 week old mice. Standard deviations are shown in the parentheses. None of the averages differed significantly ($P>0.05$) when tested by the Student *t*-test. These results indicate that infant mice used in my studies had the ability to produce IFN-γ to a similar extent as adult mice.

Effect of IFN-γ Gene Knock-out on Fungal Invasion and Tissue Damage of Mice Infected by Intragastric Route. Although I have shown that GKO mice did not have more *Candida* colonized in their GI tract, it is not known if GKO mice had more fungal invasion and tissue damage of their GI tract. GMS-stained mouse stomach CAF regions of both GKO and WT mice, which had been inoculated i.g. with *C. albicans* for 21 days, showed hyphae and yeast (data not shown) forms of *C. albicans* in the keratinized layer, but there was little evidence of fungal invasion in either the GKO or WT mice (Fig. 8A and 8B). Examination of H & E stains of the same areas showed inflammatory cell infiltration primarily at the keratinized layer where the fungi were located (Fig. 8C and 8D). For both types of mice, the infiltration was similar and consisted of 75-95% neutrophils, depending on the high powered field that was examined. Inflammatory cells were also noted, but to a lesser extent, in the lamina propria and submucosa areas immediately below the fungal-associated

keratinized layer in both kinds of mice (data not shown). There was no apparent difference in tissue damage and inflammatory cell infiltration between GKO and WT mice in any of the sections examined. There was no evidence of fungal invasion or tissue damage in the jejunum of either GKO or WT mice (data not shown).

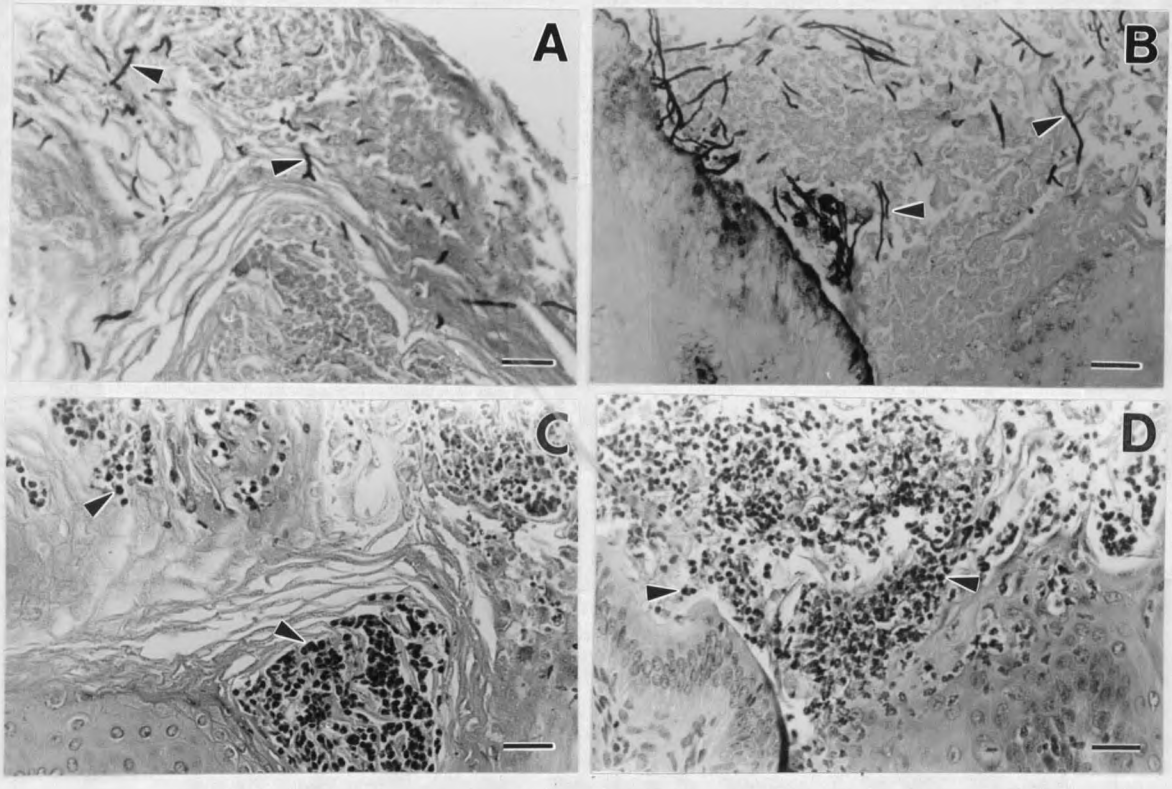


Figure 8. Histology studies of the stomach CAF region of GKO and WT mice infected with *C. albicans* intragastrically. GKO and WT mice were infected i.g. with $2x10^8$ *C. albicans* yeast cells, and 21 days later mice were sacrificed and their stomachs were removed, fixed in buffered formalin and processed for GMS (A and B) and H & E staining (C and D). A and C are tissues from GKO mice, and B and D are from WT mice. Arrows in A and B show hyphae in the tissue, and in C and D show inflammatory cells. Bar, 100 μm.

Effect of IFN-γ Gene Knock-out on *C. albicans*-Induced IA Antigen Expression by Peritoneal Macrophages. As shown in Table 8, *C. albicans* induced WT mouse peritoneal macrophages to express MHC class II IA antigen. The percentage of IA positive cells induced by *C. albicans* was about twice as many as DPBS-treated controls. However, in GKO mice, *C. albicans* was not able to induce peritoneal macrophages to express detectable levels of the IA antigen.

Table 8. *Candida* induced IA antigen expression on peritoneal macrophages of GKO and WT mice[a]

| | *C. albicans* | PBS |
|---|---|---|
| GKO | 22 | 12 |
| | 21.5 | 21.5 |
| WT | 48 | 21 |
| | 61.5 | 23 |
| | 57 | 22.5 |
| | 54 | 33 |

[a] GKO and WT mice were given i.p. $5x10^5$ *C. albicans* yeast cells or PBS control. Four days later, peritoneal macrophages were obtained, stained by immunoperoxidase staining for IA antigen expression, and 200 cells/mouse sample were counted. Values are percentage of IA positive cells from individual mice. Percentage of IA positive macrophages are significantly higher ($P<0.001$) in *Candida* infected normal mice than PBS control mice, but in GKO mice, *Candida* was not able to induce macrophages to express IA antigen.

## Discussion

Results from in vitro studies have suggested that IFN-γ may be important in host defense against disseminated candidiasis, but in vivo studies have yielded conflicting

conclusions. Differences in experimental design may explain, in part, the controversy. For example, Kullberg, et al. (146) gave mice $10^5$ units of IFN-γ by an i.v. route, while Garner, et al. (103) gave i.p. $10^2$ to $5x10^5$ units of IFN-γ to mice. The former showed decreased susceptibility to the disease, but the latter experiment resulted in increased susceptibility. Similarly, mice had increased susceptibility to the disease after they were given i.v. an anti-IFN-γ monoclonal antibody (42), while mice given the antibody i.p. showed increased resistance (123).

GKO mice provide a good tool to assess the essential role of IFN-γ in host defense against infectious diseases. Previous studies demonstrated the utility of the GKO mouse in showing the importance of IFN-γ in host defense against *Mycobacterium tuberculosis* (96) but not against *Schistosoma mansoni* (3). My data show that GKO mice are not more susceptible to a direct i.v. inoculation of *C. albicans* than WT mice. The CFU recovered from kidneys of the GKO mice were similar to those of WT mice, and GKO mice survived as long or longer than control mice.

In humans who develop the disease, the source of *C. albicans* is believed to be mostly from mucosal surfaces of the gastrointestinal (GI) tract (263,298), where *C. albicans* is normal flora. Neutrophils and macrophages are important in host defense against disseminated candidiasis, and neutrophils accumulate at the mucosal infection sites where fungal yeast and hyphal forms are located [ (53), and our unpublished observations]. IFN-γ increases neutrophil and macrophage anti-*Candida* activity in vitro (77,174,262). To test if IFN-γ may be essential to the host in limiting *Candida* spread from mucosal sites, I used

infant mice to establish mucosal colonization of *C. albicans* (215). This model of mucosal colonization was chosen because *C. albicans* does not colonize the GI tract of normal adult mice. Cole et al. (215) reported that 5- or 6-day-old CFW mice showed evidence of disseminated disease 30 min. to 3 h after an i.g. dose of the fungus. In my studies, no dissemination was detected in GKO or WT mice (BALB/c background) at 30 min. (data not shown), 3 h, 10 days and 21 days after i.g. inoculation. The extent of fungal colonization and tissue damage in the stomach and jejunum was similar to CFW mice (53). The BALB/c strain was chosen in my studies because this is the strain of mouse used by others who showed protection or no protection by IFN-γ (42,103,233). *C. albicans*-colonized GKO mice were just as resistant as normal mice colonized with the fungus to subsequent development of disseminated disease. Both kinds of mice were colonized to a similar extent following an i.g. dose of yeast cells and both resisted dissemination from the GI tract equally as well. This conclusion is based on my observations of organ counts and histological studies of tissue areas that, according to others (215), should be the areas of interest. The results of my experiments, therefore, show that IFN-γ is not essential in host resistance against disseminated candidiasis.

It is well known that IFN-γ activates macrophages and induces the expression of MHC class II IA antigen on these cells. IA plays a fundamental role in the immunoregulatory function of macrophages, such as antigen presentation to T cells. An increase in IA bearing macrophages has been observed under most of the conditions resulting in T cell activation (276). My results showed that *C. albicans* induced peritoneal

macrophages to express IA antigen in normal mice. The percentage of total cells positive for IA increased about two-fold in *Candida*-infected mice. Similar results (a two-fold increase) have been seen in syphilitic infection (273). If the fungus is able to directly stimulate macrophages, this may explain why GKO mice do not have enhanced susceptibility to candidiasis. However, in GKO mice, *Candida* infection did not cause an increase of IA positive macrophages. This is in agreement with a report that *Bacillus* Calmette-Guerin infected GKO mice did not show a high increase of IA expression on peritoneal macrophages as compared to normal mice (67). Others have reported that IL-4 may induce IA expression on macrophages (59), but my results indicate that IFN-γ is important for this expression in *Candida*-infected mice.

My experiments do not rule out a role for IFN-γ in normal mice. A complex network of endogenous mediators, such as cytokines and chemokines orchestrates the host immune response. In a normal animal, there appears to be redundancy in the production of cytokines with overlapping functions, and these pleiotropic effects may have a protective advantage for the host. For example, not only IFN-γ but also IL-1, TNF-α and IL-4 up regulate macrophage functions (59,60,210). It has been shown that IL-1 and TNF-α augment neutrophil and macrophage anti-*Candida* activity in vitro (23,91), and IL-1 had a protective effect against disseminated candidiasis in vivo (179). IFN-γ may protect the host by enhancing IL-1 or TNF-α production (104,225), and this could be compensated by other cytokines, for example, IL-2 (87,198), that induces IL-1 or TNF-α production. IFN-γ may also protect the host by direct augmentation of phagocyte anti-*Candida* functions (174).

Perhaps in the absence of IFN-γ, phagocyte anti-fungal activity is increased by other cytokines, such as GM-CSF (256), IL-1 and TNF-α (23,91).

In conclusion, my data show that IFN-γ is not essential in host defense against *C. albicans* that originates from a mucosal site or when the fungus is given directly into the bloodstream.

# Chapter 4

## CONCLUSIONS

1. Intravenous treatment of mice with liposome encapsulated dichloromethylene diphosphonate specifically deplete splenic macrophages, and possibly Kupffer cells, but does not affect neutrophils with respect to both numbers and functions.

2. An increase in susceptibility to disseminated candidiasis was found in macrophage-depleted mice as compared with macrophage-sufficient animals, even when neutrophils in the macrophage-depleted mice appeared normal. My results strongly support the idea that macrophages are important in host defense against experimental disseminated candidiasis.

3. Macrophage-depleted athymic nude mice were more susceptible to disseminated candidiasis. This observation suggests that thymus derived T cells are not involved in this macrophage protection mechanism.

4. IFN-γ gene knock-out mice were not more susceptible to either gastrointestinal originated or hematogenously disseminated candidiasis. My data suggest that IFN-γ is not essential in host defense against disseminated candidiasis.

5. IFN-γ is critical for *Candida* induced IA antigen expression by peritoneal macrophages.

# APPENDICES

# APPENDIX A

## SUSCEPTIBILITY OF SPLENECTOMIZED MICE TO DISSEMINATED CANDIDIASIS

It is widely believed that the spleen plays an important role in host defense against infections (97,167). The spleen has dual functions: filtration and immunologic destruction of invading microorganisms and the host aged cells and debris, and processing antigens. Particles in the blood may be removed by phagocytic cells in this organ (239). The role of the spleen in host defense against disseminated candidiasis is not clear. I demonstrated (see chapter 2) that elimination of mouse splenic macrophages correlates with increased susceptibility to experimental disseminated candidiasis. However, the L-$Cl_2$MDP treatment also depleted liver macrophages (Kupffer cells) (48,281,283). McCarthy, et al. (180) also reported that the mortality rate of splenectomized mice, which were given i.p. *C. albicans*, was significantly higher than that of control mice. The limitation of this work is that the mice were given the fungus immediately after the operation. At this time point, the mice may not have recovered from the operation, and the host stress response may be different between splenectomized and sham operated mice. In order to extend observations on the role of spleen in host defense against this disease, I studied disseminated candidiasis in splenectomized mice by i.v. challenge with *C. albicans* 4-5 weeks after the operation.

Four to nine week-old BALB/cBy mice were anesthetized by giving i.p. 40-80 mg/1,000 g body weight sodium pentobarbital (Sigma Chemical Co.) diluted in saline. The animals were shaved to remove the hair over the abdomen. The exposed skin was cleaned with iodine and 70 % ethanol. An incision about 2 cm long was made on the ventral midline over the abdominal area. The linea alba was then cut and the spleen was exposed. The splenic artery and vein were ligated with a single absorbable suture, and the spleen was

removed. The linea alba was sutured and the skin was sealed by appropriate application of Nexaband® liquid (Veterinary Products Laboratories, Phoenix, AZ). For sham operation controls, the procedure was the same but without the blood vessel ligation and organ removal. Four to five weeks after the operation, splenectomized, sham operated controls and normal control mice at 5 mice per group were given i.v. $2.5x10^5$ or $5x10^5$ *C. albicans* Ca-1 (see chapter 2), and survivors were observed for up to 36 days.

As shown in Fig. 9, at different concentrations of *C. albicans* inoculation, splenectomized mice were not more susceptible than sham operated and normal control mice ($P>0.05$, Mann-Whitney test). It is difficult to make conclusions at this point because these experiments are limited. I challenged mice at only one time point after splenectomy. The 4-5 week period was chosen as based on malaria studies in mice (247). Perhaps the animals should be evaluated for susceptibility to candidiasis well before that time. By 4 weeks after splenectomy compensatory mechanisms may become activated, such as elevated NK cell numbers (93).

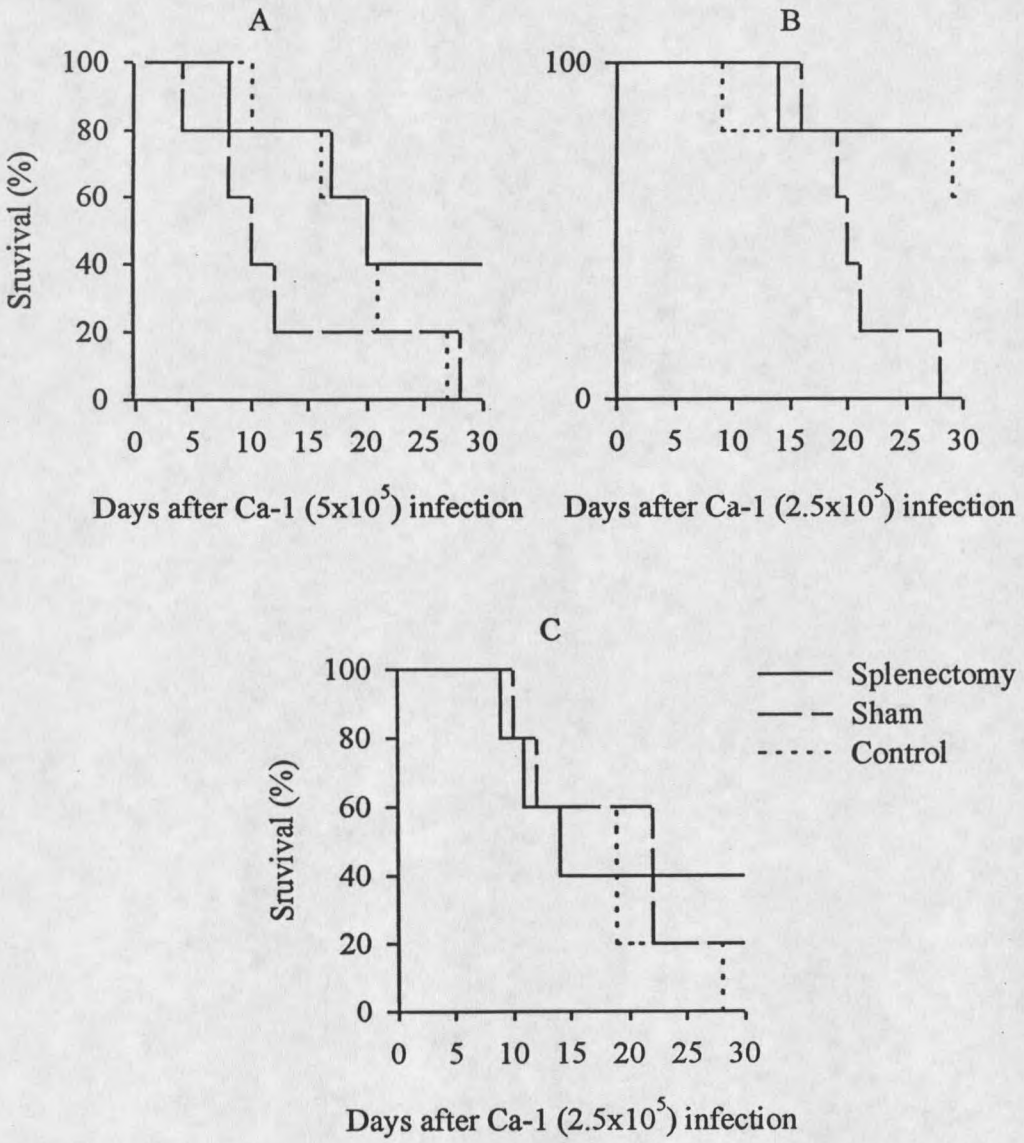


Figure 9. Susceptibility of splenectomized mice to disseminated candidiasis. BALB/cBy mice were splenectomized or sham operated. Four to five weeks later, the mice were given i.v. $5x10^5$ (A) or $2.5x10^5$ (B and C) *C. albicans* Ca-1 yeast cells. The number of survivors as a function of time was recorded. The data were from two separate experiments, experiment 1 (A and B) and experiment 2 (C).

# APPENDIX B

## SUSCEPTIBILITY OF NEUTROPENIC MICE TO DISSEMINATED CANDIDIASIS

As discussed in chapter 1, neutrophils are important in host defense against disseminated candidiasis. When CB.17 and *scid* mice were given mAb RB6-8C5, which eliminates murine granulocytes, the mice were more susceptible to i.v. challenge with *C. albicans* (124,125). The numbers of *Candida* CFU recovered from liver, spleen, and kidneys of neutropenic mice were significantly higher than that of control mice. To compare the effect of macrophage depletion to neutrophil depletion in host defense against disseminated candidiasis, I did the following experiments.

When mice were treated i.p. with a single dose of RB6-8C5 mAb, they became severely neutropenic by 24 h after the treatment and remained neutropenic for up to 5 days (124). To verify the neutrophil depletion in my experiments, I counted circulating and peritoneal elicited neutrophils. BALB/cBy mice were given either i.p. 250 μg/mouse mAb RB6-8C5 (a kind gift from Mark A. Jutila, Montana State University) or PBS as a control. Twenty-four hours later, peripheral blood was obtained from the tail artery of each mouse for differential counts, and each mouse was given i.p. 1 ml of thioglycollate (BBL Microbiology Systems, Becton Dickinson and Co. Cockeysville, MD). Four hours later, the mice were sacrificed, and the peritoneal cavity of each mouse was lavaged with 8 ml of Hanks' balanced salts solution without $NaHCO_3$, $Ca^{++}$, and $Mg^{++}$ (GIBCO, Grand Island, NY.) containing 0.05M EDTA (HBSS). The peritoneal elicited cells were pelleted by centrifugation and cell smears were made on glass slides. The slides were stained by Wright stain, and 200 cells per slide were counted. The spleen of each mouse was removed for immunoperoxidase staining for neutrophils as described in chapter 2.

As shown in table 9, the percentage of neutrophils in peripheral blood of mAb RB6-8C5 treated mice was minimal, but that of control mice was about 18%. The percentage of neutrophils in thioglycollate elicited peritoneal cells of mAb treated mice was also minimal (table 9), while that of control mice was about 65 %. The immunoperoxidase staining for neutrophils showed insignificant staining of the spleens from mAb RB6-8C5 treated mice, but strong staining of spleens from control mice (data not shown).

Table 9. Peripheral blood and elicited peritoneal cell differential counts of neutropenic and normal mice.[a]

| | | Cell Differential Count (%) | | | | |
|---|---|---|---|---|---|---|
| | Treatment | Neutrophil | Lymphocyte | Monocyte | Eosinophil | Basophil |
| Blood | RB6-8C5 | 0.3±0.6[b] | 98±0.5 | 1.2±0.8 | 0.5±0.5 | 0 |
| | PBS | 18.3±0.3 | 79.8±0.8 | 0.5±0.5 | 1.3±0.6 | 0 |
| Peritoneal | RB6-8C5 | 0.7±0.6 | 98.3±1.6 | 0.5±0.9 | 0.3±0.6 | 0.17±0.3 |
| | PBS | 65.5±5.9 | 34.5±5.9 | 0 | 0 | 0 |

[a] Mice were given RB6-8C5 or PBS, 24 h later, blood was obtained from the tail artery, and differential counts were made. Mouse was then given i.p. 1 ml of thioglycollate, and 4 h later, mice were sacrificed. Peritoneal elicited cells were collected and differential counts were made.
[b] Mean value and standard deviation of three mice.

To compare the susceptibility of mice depleted of macrophages or neutrophils to disseminated candidiasis, mice (5 mice/group) were treated as follows: at day 0, two groups of mice were given i.v. either 0.2 ml L-$Cl_2$MDP or PBS (see chapter 2); at day 2, two other groups of mice were given i.p. either 250 µg/mouse mAb RB6-8C5 or PBS; at day 3, all

mice were given i.v. $5x10^5$ *C. albicans* Ca-1 yeast cells prepared as described in chapter 2, and observed for survivors for up to 25 days.

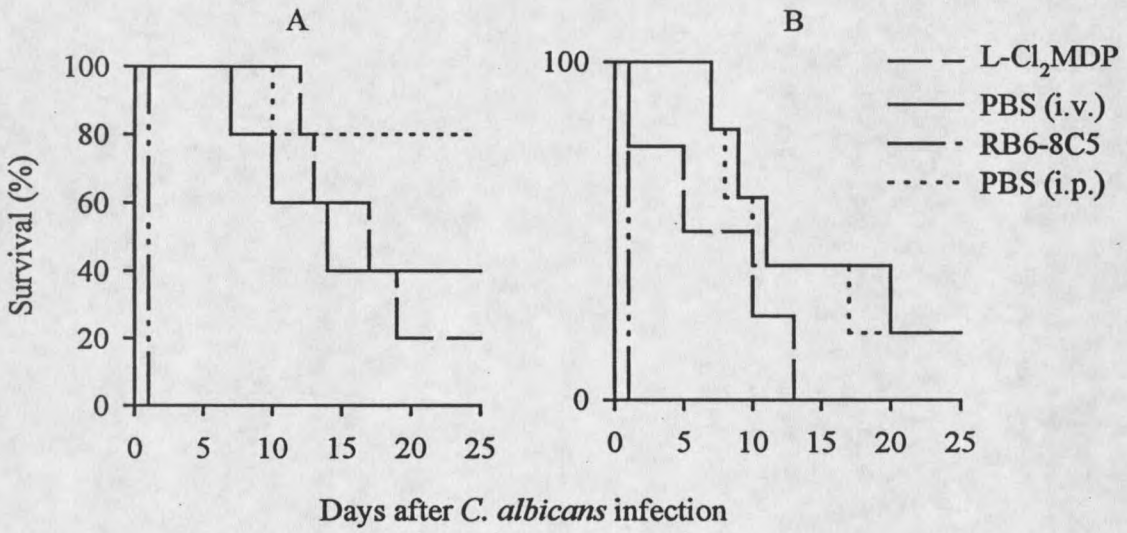


Figure 10. Susceptibility of mice depleted of macrophages or neutrophils to disseminated candidiasis. *Scid* (A) and BALB/cBy (B) mice were depleted of macrophages or neutrophils by treatment with either L-$Cl_2$MDP i.v. or mAb RB6-8C5 i.p. Three days after L-$Cl_2$MDP treatment or 24 h after RB6-8C5 treatment, mice were given i.v. $5x10^5$ *C. albicans* Ca-1, and observed for survivors for 25 days.

The neutropenic *scid* mice were more susceptible to disseminated candidiasis than macrophage-depleted mice and control mice (fig. 10) ($P<0.01$, Mann-Whitney test). The neutropenic BALB/c mice were more susceptible than control mice ($P<0.01$). The neutropenic mice showed a similar tendency to be more susceptible to the disease than the macrophage depleted mice ($P>0.05$). Infecting BALB/c mice with $2x10^5$ *C. albicans* may show a significant difference between neutropenic and macrophage depleted mice. This suggests that neutrophils are more critical than macrophages in host defense against the

disseminated disease in these mice. The macrophage depleted *scid,* and BALB/c mice were not more susceptible to the disease than control mice in this experiment ($P>0.05$), but this study was done on mice challenged with only one concentration of *Candida.* Giving different numbers of *C. albicans* to mice and then observing survivors may be valuable for further studies. I have shown in Chapter 2 that macrophage depleted BALB/c mice were more susceptible to disseminated candidiasis than control mice when they were given i.v. $2x10^5$ *C. albicans* yeast cells.

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