In vivo effect of mouse peritoneal cells on Candida albicans
by Anne Holly Poor

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE
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Abstract:
Millipore diffusion chambers containing the fungus Candida albicans were implanted in the peritoneal cavity of normal and nude BALB/c mice to study the effects of host soluble and cellular factors on the growth of C. albicans. Opsonins, 51Cr solution, and 51c r-labeled neutrophils were able to enter chambers which were implanted in the peritoneal cavity 2k hr earlier but were unable to enter chambers which were present in the peritoneal cavity for 7 days. Fungal growth in vivo was always greater in chambers that admitted soluble factors but excluded host cells (small porosity chambers) than in chambers that admitted soluble factors and also allowed emigration of host cells; however growth seen in chambers in vitro was several orders of magnitude greater than growth seen in vivo. Peritoneal exudate cells (PEC) were added directly to small porosity chambers with C. albicans to study the effects of specific cell populations on the fungus. Neutrophi 1-rich PEC harvested from normal mice were candidacidal at high neutrophil to yeast ratios, while macrophage-rich PEC were candidastatic at all macrophage to yeast ratios tested. Neutrophi 1-rich and macrophage-rich PEC harvested from nude mice both were candidacidal at high phagocyte to yeast ratios.

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Date Aug. 20, 1979

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by

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A thesis submitted in partial fulfillment
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TABLE OF CONTENTS

	Page
VITA	ii
ACKNOWLEDGMENTS	iii
TABLE OF CONTENTS	iv
LIST OF TABLES	vi
LIST OF FIGURES	vii
ABSTRACT	viii
INTRODUCTION	1
MATERIALS AND METHODS	8
Source of experimental animals	8
Yeasts and growth conditions	8
Construction of diffusion	9
Filling and implantation of chambers	9
Harvest of chamber contents and assay for viable *C. albicans*	10
Preparation of peritoneal exudate cells (PEC)	10
Serum source	12
Preparation and use of ^{51}Cr labeled cells	12
Phagocytic cell function tests	13
RESULTS	15
Fate of C. albicans in diffusion chambers *in vivo* and *in vitro*	15

Page

Treatment of the fibrin clot inside chambers 20

Emigration of host peritoneal cells into 3p chambers 20

Entry of opsonic factors into diffusion chambers 24

Effect of neutrophil-rich PEC on C. albicans in diffusion chambers 25

Effect of macrophage-rich PEC on C. albicans in diffusion chambers 29

DISCUSSION 33

LITERATURE CITED 44

APPENDIX I Effect of neutrophils on Streptococcus pneumoniae in diffusion chambers 52

APPENDIX II Effect of silica on the phagocytic ability of mice 56

APPENDIX III Effect of phagocytic cells from normal mice on C. albicans in diffusion chambers in the presence of immune serum 61

REFERENCES TO APPENDICES 64

LIST OF TABLES

Table Page

1. Growth of *C. albicans* in diffusion chambers *in vitro* at 37°C 19

2. Effect of homogenizing filters and chamber contents on the viability of *C. albicans* in diffusion chambers 21

3. *In vivo* opsonization of *C. albicans* in diffusion chambers 26

4. Effect of neutrophils harvested from normal mice on *C. albicans* in diffusion chambers 27

5. Effect of neutrophils harvested from nude mice on *C. albicans* in diffusion chambers 28

6. Effect of macrophages from normal mice on *C. albicans* in diffusion chambers 30

7. Effect of macrophages harvested from nude mice on *C. albicans* in diffusion chambers 32

8. Effect of neutrophils on *Streptococcus pneumoniae* in diffusion chambers 54

9. Clearance of *Candida albicans* from blood of normal and silica treated BALB/c mice 59

10. Effect of silica on the phagocytic ability of BALB/c mice 60

11. Effect of phagocytic cells from normal mice on *C. albicans* in diffusion chambers in the presence of immune serum 63

LIST OF FIGURES

Figure | Page

1. Fate of C. albicans in 0.45p and 3p diffusion chambers *in vivo* 17

2. Emigration of host peritoneal cells into 3p diffusion chambers 23

ABSTRACT

Millipore diffusion chambers containing the fungus *Candida albicans* were implanted in the peritoneal cavity of normal and nude BALB/c mice to study the effects of host soluble and cellular factors on the growth of *C. albicans*. Opsonins, ^{51}Cr in solution, and ^{51}Cr-labeled neutrophils were able to enter chambers which were implanted in the peritoneal cavity 24 hr earlier but were unable to enter chambers which were present in the peritoneal cavity for 7 days. Fungal growth *in vivo* was always greater in chambers that admitted soluble factors but excluded host cells (small porosity chambers) than in chambers that admitted soluble factors and also allowed emigration of host cells; however growth seen in chambers *in vitro* was several orders of magnitude greater than growth seen *in vivo*. Peritoneal exudate cells (PEC) were added directly to small porosity chambers with *C. albicans* to study the effects of specific cell populations on the fungus. Neutrophil-rich PEC harvested from normal mice were candidacidal at high neutrophil to yeast ratios, while macrophage-rich PEC were candidastatic at all macrophage to yeast ratios tested. Neutrophil-rich and macrophage-rich PEC harvested from nude mice both were candidacidal at high phagocyte to yeast ratios.

INTRODUCTION

Candida albicans is a ubiquitous yeast-like fungus which is the most common etiologic agent of the disease candidiasis. This organism is considered to be an opportunistic pathogen because individuals who get candidiasis are compromised in defense against infection. Candidiasis is a complex disease: it can occur in the human at many sites depending on the severity of the underlying predisposing disorder. The prognosis and therapy of candidiasis varies depending on the site of infection.

Human candidiasis can present in many forms. One form of candidiasis is a simple cutaneous infection. The predisposing factor is generally an abnormality that results in a local loss of innate defense, such as in burns or as in the neonate's lack of normal flora. This form of candidiasis usually improves when the predisposing factor is eliminated, otherwise topical antibiotic therapy may be necessary. Chronic mucocutaneous candidiasis (CMCC) is an uncommon disease which is characterized by a widespread infection of skin, nails, and mucous membranes. Patients with this disease usually have an underlying defect in T cell function. Intravenous therapy with the antimycotic amphotericin B provides only transient remission. Systemic candidiasis has become recognized as a common nosocomial infection; it occurs in patients who are severely debilitated for example by therapeutic immunosuppression, neoplasia, or treatment with broad spectrum anti-

biotics. (21). In systemic candidiasis, it is thought that the fungus disseminates hematogenously from a portal of entry, which is most often the gastrointestinal tract (33,23) but may also be the lung (48). Foci of infection can be seen in different internal organs in a single patient. The lung, liver, spleen, or brain may be affected, however the kidney is the organ most commonly affected. Amphotericin B therapy is essential for recovery from systemic candidiasis.

Clinical observations and experimental animal models point to several aspects of immunity which are important in resistance to candidiasis. Human clinical cases exist which have brought some investigators to suggest that T cell dependent cell mediated immunity (CMI) is important in defense against candidiasis. Individuals with congenital thymic aplasia, such as DiGeorge and Nezelof-Allibone syndromes are more susceptible than normal people to CMCC. Other individuals who have CMCC also exhibit abnormalities in T cell related functions, for example cutaneous anergy to Candida antigen, deficient in vitro lymphocytic transformation responses, or deficient synthesis of macrophage inhibition factor (MIF) after exposure to Candida antigen (21,33,64).

Experimental animal correlates of human disease which also suggest that CMI is important in resistance to candidiasis include a murine model of cutaneous candidiasis and NZB mice. Domer, et al.

found that delayed-type hypersensitivity to cell wall and cell membrane antigens develops after cutaneous infection in mice (20). Other workers found that NZB mice, known to develop abnormalities in T cell dependent function with age, are more susceptible to an intravenous dose of _C. albicans_ than are other strains of mice tested (13).

In addition to studies which suggest that T cells are important in host defense to candidiasis, evidence exists which suggests that phagocytic cells are also important. Biopsy material from human lesions may show an acute inflammatory response surrounding foci of _Candida_ infection, a response consisting predominantly of neutrophils (43). In patients with _Candida_ meningitis an intense pleocytosis of the spinal fluid has been noted, which is like a pyogenic response to bacterial infections (66). The attraction of neutrophils to an area of _Candida_ infection may occur due to generation of chemotactic factors either by activation of the alternative pathway of complement by _Candida_ cell walls (52), or possibly, by metabolic products of the fungus (15). Defective leukocyte chemotaxis is seen in patients with severe burns, this correlates with their increased susceptibility to _Candida_ infections (65). The importance of neutrophils in prevention of dissemination of _Candida_ is suggested in clinical studies which show that granulocytopenia is a major predisposing factor leading to _Candida_ sepsis (45,51). Humans with Chediak-Higashi syndrome have

neutrophils that display abnormal morphology, defective migration, and defective chemotaxis (67). Although these patients usually suffer from pyogenic infections but not candidiasis, a murine correlate of human Chediak-Higashi syndrome (26) exhibits an increased mortality rate over normal mice when infected with C. albicans (22).

In vitro studies form a number of laboratories have substantiated the ability of human neutrophils and macrophages to phagocytize and kill C. albicans (47,37,4). Women who suffered recurrent attacks of vaginal candidiasis had leukocytes that displayed normal ingestion but deficient killing of C. albicans in vitro (58). A previously healthy young girl with disseminated candidiasis had a leukocyte defect that was specific for intracellular killing of C. albicans (61). In one study (64), 40% of CMCC patients studied had normal CMI; one may speculate that these patients had CMCC because of defects in innate immunity. Clinical studies which support this speculation include in vitro experiments where granulocytes from CMCC patients had phagocytic and candidacidal defects (19), and other studies where mononuclear cells from a CMCC patient had defective chemotactic responses (60). In vitro studies by Lehrer and Cline (34-36) demonstrated that leukocytes from chronic granulomatous disease patients and from patients with hereditary myeloperoxidase deficiency were unable to kill C. albicans, indicating that myeloperoxidase and H_2O_2 are necessary in the candidacidal mechanism.

Other experimental animal models exist which suggest that innate immunity is important in defense against candidiasis. Thymectomized mice (29) and congenitally athymic (nude) mice (14,54) are more resistant to certain forms of acute infections by _C. albicans_. In murine models of cutaneous candidiasis abscesses develop which contain high numbers of neutrophils (28,49). In one study, recovery from candidiasis and resistance to reinfection by _C. albicans_ occurred without histopathological indicators of CMI (49). Corticosteroid treatment of mice was found to potentiate candidiasis and renal _Candida_ lesions in these mice were deficient in inflammatory cells (7). In another study, reduced accumulation of neutrophils at foci of _Candida_ infection was correlated with increased susceptibility of mice to candidiasis when mice were previously injected with L1210 leukemia cells (32). Other workers studied the resistance to candidiasis in experimental animals depleted of certain chemotactic factors. Guinea pigs that were treated with cobra venom factor (CVF) to deplete C3 (53) exhibited increased mortaliity when challenged with _C. albicans_ as compared to control animals which were not treated with CVF (27).

Evidence cited in the above paragraphs indicates that innate immunity plays a role in defense against candidiasis. Other workers have investigated the effect of human (34,37,47) and animal (4) phagocytic cells on _C. albicans in vitro_. Since results of _in vitro_

systems can not always be readily interpreted to apply to *in vivo* situations, the goal of this project was to study the effect of phagocytic cells on *C. albicans in vivo*. This work involved implanting Millipore diffusion chambers containing *C. albicans* in the peritoneal cavity of mice to study the candidacidal effects of phagocytic cells *in vivo*.

Diffusion chambers implanted in the peritoneal cavity of mice represent a controllable *in vivo* environment in which cells and cell-host interactions may be studied. Diffusion chambers have been used *in vivo* in the past to study tumor cell growth (1) and host immunity to tumors (2). More recently, diffusion chambers have been used *in vivo* to culture mouse (6,63,11,10,5,9) and human (24,31,8) hematopoeitic cells, to study the regulation (56,52) and kinetics (46) of antibody synthesis by lymphoid cells, and to study the effects of anticancer drugs on various human tumors (40).

In my experiments, *C. albicans* was added to diffusion chambers which were then implanted in the peritoneal cavity of mice. The porosity of the membrane filters composing the sides of the chambers was such that the fungal cells could not escape the chambers. The effect of soluble factors and emigrating peritoneal cells on *C. albicans* in diffusion chambers was examined by constructing the chambers with membrane filters of various porosities. Also, the

effect of specific phagocytic cell populations was examined by adding peritoneal cells directly to chambers with C. albicans. Chambers could then be later removed from the peritoneal cavity and assayed for viable C. albicans.

MATERIALS AND METHODS

Source of experimental animals. BALB/c mice, originally obtained from Baylor Medical School (Houston, TX), were maintained on food (Wayne Lab Blox Allied Mills Inc., Chicago, IL) and acidified water (41) *ad libidum*. Cages and bedding were sterilized prior to use. Fecal specimens periodically plated onto blood agar, sabouraud-dextrose agar and Mycosel agar (BBL, Cockeysville, MD) were negative for *Candida albicans*. Sera obtained from randomly selected mice were negative for *Candida* agglutinins and precipitins (14).

Congenitally thymus-deficient (nude) mice were raised together with their phenotypically normal littermates (NLM; +/+, or nu/+) in separate quarters. Nude mice were from a colony in which cross-intercross mating is in progress to derive a line congenic with BALB/c mice. Hereafter BALB/c mice and NLM are designated as normal mice.

Yeasts and growth conditions. *Candida albicans* strain 9938 (Medical Mycology Unit, Tulane University) was used in all experiments. The yeasts were grown aerobically in 2.0% glucose-0.3% yeast estract-1.0% peptone broth. The cultures were rotated at 160 rpm (Gyrotatory Incubator-Shaker, New Brunswick, NJ) for 48-72 hr at 37°C. Organisms were harvested by centrifugation, washed three times in sterile saline, and resuspended to the appropriate concentration in 0.01M phosphate buffered saline, pH 7.2 (PBS). Yeast viability was determined to be at least 95% by plating suspensions of known concentration onto

sabouraud-dextrose agar.

Construction of diffusion chambers. Millipore membrane filters (13 mm diameter), type HA of 0.45 µm porosity (0.45 p), or type SS of 3 µm porosity (3 p) (Millipore Corp., New Bedford, MA) were glued (Millipore Cement #1) to each side of 3 mm thick Millipore lucite rings and the glue was allowed to dry for at least 8 hr before use at 23-25°C. Some chambers were checked for the presence of leaks by blowing air into chambers under water as advocated by Amos (3). The chambers were surface decontaminated by exposure of each side to uv irradiation for 20 min in a safety-hood (Bioquest, Cockeysville, MD); these conditions were previously determined to be microbicidal.

Filling and implantation of chambers. Chambers were placed between the tips of goose-neck forceps, and 0.15 ml of the cell suspension (either *C. albicans* alone or *C. albicans* together with mouse peritoneal cells) was added to each chamber with a syringe and 25 gauge needle through a radially located hole in the lucite ring. The hole was sealed with a small amount of parafin (Sherwood Medical Industries, St. Louis, MO). Normal or nude mice were anesthetized with 0.06 mg/g body weight sodium pentobarbital (50) and the chambers were implanted into the peritoneal cavity through a ventral near-midline incision which was then closed with surgical clips (Autoclips, Clay Adams,

Parsippany, NJ).

Harvest of chamber contents and assay for viable *C. albicans*. Mice were killed by cervical dislocation and the chambers were removed and added to sterile distilled water for 5 min. The filters were cut from the lucite rings with a scalpel and homogenized with the chamber contents and 5 ml of sterile saline in a glass tissue homogenizer. Dilutions of the homogenates were plated on sabouraud-dextrose agar containing 50 units/ml penicillin and 50 μg/ml streptomycin (Microbiological Associates, Bethesda, MD). Colony forming units (cfu) of *C. albicans* were counted after 36 hr incubation at 37°C.

Preparation of peritoneal exudate cells (PEC). A neutrophile-rich PEC population was obtained from 12-24 week old male or female normal or nude mice. Mice were injected intraperitoneally (ip) with 3 ml of 0.5% glycogen (Nutritional Biochemicals, Cleveland, OH) in saline. Four to six hours later the mice were killed by cervical dislocation, the abdominal skin was reflected and a small incision was made in the peritoneal wall. The peritoneal cavity was washed out with approximately 5 ml of Hank's Balanced Salts Solution (HBSS) (GIBCO, Grand Island, NY) containing 0.002M disodium ethylene-diamine-tetraacetate (EDTA). The washes were centrifuged at 200 x g for 10 min, the cells were washed once with HBSS, and resuspended in PBS to the appropriate

concentration. The cell population was at least 85% neutrophils as determined by examination of acetic acid wet mounts, which cleared the cells so that the nuclear morphology was evident, and by Giemsa stained smears. At least 90% of the cells were viable as determined by trypan blue exclusion.

A macrophage-rich PEC population was obtained by injecting normal or nude mice ip with 3 ml of NIH thioglycolate (Difco, Detroit, MI). The PEC were harvested 3 days later and cell suspensions were prepared as described above. The cell population was 60-80% macrophages as determined by examination of Giemsa-stained smears, and at least 85% of the cells were viable.

PEC collected from thioglycolate-stimulated BALB/c mice were further enriched for macrophages by ficoll density gradient centrifugation using a method described by Zambala and Asherson (69). About 10^8 peritoneal cells were suspended in 4 ml of 25% ficoll (Pharmacia, Uppsala, Sweden) in Balanced Salts Solution (BSS) (42) and placed in a 30 ml thick-walled glass centrifuge tube (Corning, Corning, NY). The cells were overlaid with 6 ml of 21%, 5 ml of 16% and 4 ml of 12% ficoll solution and centrifuged in a swinging bucket rotor (Sorvall HB-4) at 20,000 x g for 70 min at 4°C. The cells in the top 2 interfaces were collected with a Pasteur pipette, washed twice with BSS, and suspended to the proper concentration in PBS.

Serum source. In some experiments, the peritoneal cells were suspended in homologous serum rather than PBS. Normal BALB/c mice were lightly anesthesized with ether and bled from the orbital venous plexus, the blood was allowed to clot at 4°C. The serum was used within 2 hr or stored at -20°C until use.

Preparation and use of ^{51}Cr labeled cells. Sodium chromate (^{51}Cr) (New England Nuclear, Boston, MA) of specific activity 300-500 mCi/mg Cr was used to label neutrophil and spleen cell populations using a method described by others (25). Neutrophil-rich PEC were obtained as described above. Spleen cells were obtained from normal BALB/c mice. The mice were killed by cervical dislocation, an incision was made through the peritoneal wall, and the spleen was removed and added to 5 ml of HBSS+EDTA. A single cell suspension was made by gently rubbing the spleen against a small gauge aluminum screen. The suspension was centrifuged at 200 x g for 10 min, and the cells were washed once with PBS. The cells were determined to be at least 80% viable by trypan blue exclusion. The neutrophil and spleen cell populations were suspended to about 2×10^8 cells/ml in PBS containing 15% fetal calf serum (FCS) (Microbiological Associates) and 50-75 mCi of ^{51}Cr were added per ml cell suspension. The cells were incubated at 37°C in a humidified chamber under 5% CO_2 for 1 hr, mixed every 15 min, then collected by centrifugation at 200 x g for 10 min and washed

twice with PBS. Approximately 1 x 10^8 labeled PEC or spleen cells were injected intravenously (iv) into normal mice implanted with 3 p chambers containing 5 x 10^4 _C. albicans_. Chambers were harvested 6 hr after the iv injection of ^{51}Cr labeled cells and rinsed in PBS. Filters and chamber contents were counted separately for 1 min each in a gamma counter (Beckman Biogamma, Beckman Instruments, Fullerton, CA), at window settings of 0 (lower) and 300 (upper). The counting efficiency of this machine was 72% under these conditions. In some experiments, the chamber contents were centrifuged at 200 x g for 10 min and the supernatent liquid and cell pellet were counted separately.

Phagocytic cell function tests. An _in vitro_ phagocytic assay described by others (44) was used to test phagocytic functions of peritoneal cells. Chambers of 0.45 µm porosity containing 1 x 10^8 heat killed yeasts were harvested 24 hr after implantation; the yeasts were recovered by vortexing the filters and chamber contents. PEC were collected from unstimulated normal mice and suspended in medium 199 (M199) (GIBCO); 0.5 ml of this suspension (2 x 10^6 cells/ml), with 5% NMS or NMS absorbed 3 times with 1/10 volume of _C. albicans_ cells at 37°C (INMS) was added to 22 mm x 22 mm cover glasses (VWR Scientific, Seattle, WA) and incubated in a humidified chamber at 37°C for 1 hr. The cover glasses were rinsed with 2 ml of HBSS. One-half ml of the _C. albicans_ suspension obtained from the chambers or 0.5 ml of a heat

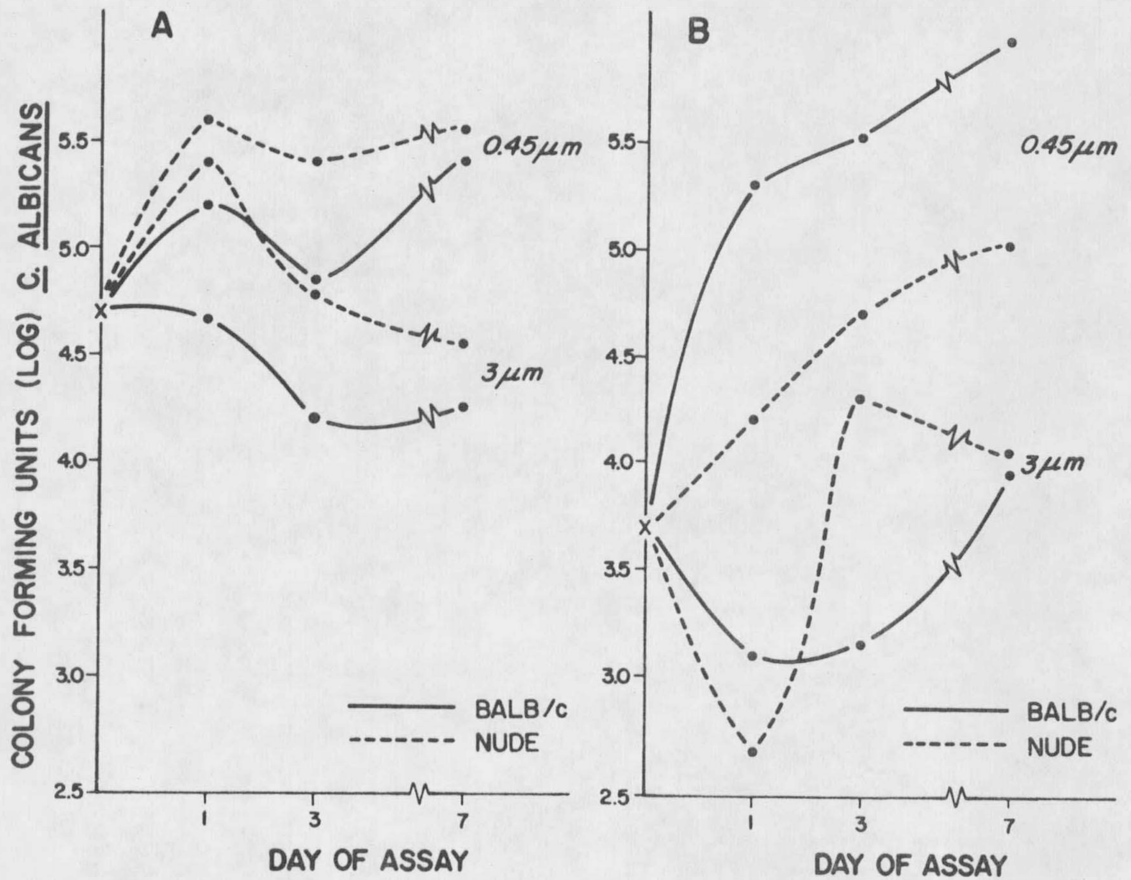
killed *C. albicans* suspension containing 4 x 10^6 yeast cells per ml with 5% NMS or INMS was added to the cover glasses containing the macrophages and incubated at 37°C for 1 hr. The cover glasses were washed with 2 ml of HBSS, air dried, fixed with methanol for 3 min, stained with Giemsa, and examined using light microscopy at 1000X. All tests were done in duplicate and 200 macrophages per cover glass were counted. The percent phagocytosis represents the mean percentage of macrophages that had ingested one or more yeast cells.

Random migration of neutrophils was studied using the chemotaxis under agarose method (15).

RESULTS

Fate of C. albicans in diffusion chambers in vivo and in vitro. The fate of Candida albicans in vivo in the peritoneal cavity of mice was tested in 3p chambers, which allow emigration of host peritoneal cells, and in 0.45p chambers, which exclude host peritoneal cells. Various concentrations of yeast cells were added to chambers, the chambers were implanted ip into BALB/c or nude mice, and the number of viable C. albicans remaining in the chambers was assayed on days 1, 3, 5, and 7 post-implantation. When 5 x 10^4 yeasts were added to chambers which were implanted in BALB/c mice (Figure 1-A), the number of fungal units recovered from 0.45p chambers increased by about 0.75 $\log_{10}$ after 7 days of in vivo inclubation, while the number of fungal units recovered from 3p chambers decreased by 0.5 log after 7 days. Similar results were seen when chambers containing 5 x 10^4 yeasts were implanted in nude mice, except that the number of cfu recovered from 3p chambers at 7 days was approximately the same as the inoculum. Also, the number of C. albicans cfu recovered from both 0.45p and 3p chambers after 28 days of in vivo incubation was about the same as the number of cfu recovered from chambers at 7 days. When 5 x 10^3 yeasts were added to chambers (Figure 1-B) the number of cfu recovered from 0.45p chambers implanted in BALB/c mice increased over the inoculum by over 2 logs after 7 days, but increased by only about 1 log in 0.45p chambers implanted in nude mice. In contrast, C. albicans cfu recovered from 3p

Figure 1. Fate of C. albicans in 0.45μ and 3μ diffusion chambers in vivo. A - 5 x 10^4 C. albicans were added to chambers; B - 5 x 10^3 C. albicans were added to chambers.


A
B
COLONY FORMING UNITS (LOG) C. ALBICANS
5.5
5.0
4.5
4.0
3.5
3.0
2.5
0.45µm
3µm
BALB/c
NUDE
1
3
7
DAY OF ASSAY

chambers implanted in BALB/c mice decreased about 0.5 log after 1 day of *in vivo* incubation, and decreased about 1 log in nude mice. After 7 days of incubation, the number of fungal units recovered from 3μ chambers increased to slightly exceed the inoculum.

The growth of *C. albicans* in diffusion chambers *in vitro* was determined by adding various concentrations of yeasts to 0.45μ chambers, adding the chambers to 50 ml flasks containing 10 ml of GYEP, and incubating for 48 hr at 37°C. After incubation the filters and chamber contents were removed and either vortexed or homogenized in 5 ml of saline. Regardless of the size of the yeast inoculum and method of recovery (vortexing or homogenizing) the number of cfu recovered from the chambers was approximately the same (Table 1). Similar numbers of yeasts were recovered if the chambers were incubated in 10 ml of FCS rather than GYEP. To determine the accuracy of homogenizing and subsequent plating of filters and chamber contents, 0.45μ chambers containing 4×10^7 yeasts were implanted ip and harvested 48 hr later. After vortexing the filters and chamber contents in 5 ml saline, an aliquot of the supernatent liquid was assayed for the number of yeasts by direct counting using a hemacytometer and by plating onto sabouraud-dextrose agar. The same filters and chamber contents were then homogenized and plated onto sabouraud-dextrose agar. Homogenization did not decrease the viability of *C. albicans*, and plate counts of yeasts

Table 1. Growth of *C. albicans* in diffusion chambers *in vitro* at 37°C

No. yeasts added	Log_{10} cfu recovered after 48 hr	
	homogenized	vortexed
$4x10^7$	8.34 ± 0.17	8.26 ± 0.14
$5x10^4$	8.29 ± 0.26	8.29 ± 0.13

correlated with direct counts (Table 2).

Treatment of the fibrin clot inside chambers. Chambers were removed from mice after 48 hr of *in vivo* incubation and added to a flask containing 10 ml of HBSS + 5% Pronase (Sigma) which was then rotated at 150 rpm for 55 min at 22-25°C. Pronase treatment adequately dissolved the fibrin clot which forms on the inside of chambers; the number of *C. albicans* cfu recovered after Pronase treatment was similar to the number recovered after homogenization.

Emigration of host peritoneal cells into 3p chambers. 3p chambers containing yeasts were harvested 6 hr, 24 hr, or 7 days after implantation. The chamber contents were obtained by washing with saline and the number of white cells that entered the chamber was determined by counting in a hemacytometer. About 3.2 x 10^{6} peritoneal cells entered the chambers by 6 hr and their numbers remained constant for 7 days (Figure 2). Microscopic examination of Giemsa stained smears of contents of these chambers showed that in chambers harvested at 6 hr 87% of the cells were neutrophils, and in the chambers harvested at 24 hr 92% of the cells were neutrophils.

In other experiments, ^{51}Cr-labeled neutrophils, ^{51}Cr-labeled spleen cells, or free ^{51}Cr in PBS were injected iv into mice which were implanted with 3p chambers 6 hr, 24 hr, or 7 days previously.

Table 2. Effect of homogenizing filters and chamber contents on the viability of *C. albicans* in diffusion chambers

Method of recovery of yeasts		Log_{10} no. cfu *C. albicans* recovered
vortexed -	direct count	7.51 ± 0.05
	plate count	7.55 ± 0.11
homogenized -	plate count	7.62 ± 0.23

Figure 2. Emigration of host peritoneal cells into 3p diffusion chambers