



The role of serum components in the phagocytosis of *Candida albicans* by mouse peritoneal macrophages
by Richard Pearce Morrison

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE
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Abstract:

Mouse serum is required for optimum in vitro phagocytosis of *Candida albicans* by mouse peritoneal macrophages. If we used fresh mouse serum, heated mouse serum (56°C) or no serum, the percentages of macrophages that ingested *C. albicans* were 83, 22 and <5, respectively. Low phagocytic activity (<5%) also occurred if fresh serum was preabsorbed with *C. albicans* at 37°C. In attempts to identify some of the serum factors which adsorb to *C. albicans*, we found that yeast cells adsorbed with either fresh serum or heated serum were strongly agglutinated when treated with anti-C3, slightly agglutinated with anti-IgG or anti-IgA and not agglutinated with anti-IgM. Serum either pretreated with anti-C3, anti-whole mouse, Zymosan at 37°C or EDTA did not support phagocytosis. However, C5-deficient serum and serum either treated with anti-IgG or EGTA was fully active. Serum treated to remove precursors of the alternative complement pathway supported an intermediate phagocytic activity (30%). Specific *C. albicans* antibody appears not to be responsible for any level of phagocytic activity observed in our test system because passage of fresh mouse serum through an affinity column containing *C. albicans* cell wall antigens did not alter the phagocytosis promoting activity of the serum. If serum was not added to the test system but if *C. albicans* adsorbed with fresh serum was used, 14% phagocytosis was observed. But if serum preabsorbed with *albicans* was used along with yeasts adsorbed with fresh serum, a high level of phagocytosis (72%) occurred. These data indicate that two serum factors will adsorb to *C. albicans* yeast cells and are required for optimum levels of phagocytosis. Other data are presented which indicate that the two factors are C3b and native C3. A third component, which is heat stable but not adsorbed by *C. albicans* is also required for maximum in vitro phagocytic activity.

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CANDIDA ALBICANS BY MOUSE PERITONEAL MACROPHAGES

by

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A thesis submitted in partial fulfillment
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ABSTRACT

Mouse serum is required for optimum in vitro phagocytosis of Candida albicans by mouse peritoneal macrophages. If we used fresh mouse serum, heated mouse serum (56°C) or no serum, the percentages of macrophages that ingested C. albicans were 83, 22 and <5, respectively. Low phagocytic activity (<5%) also occurred if fresh serum was preabsorbed with C. albicans at 37°C. In attempts to identify some of the serum factors which absorb to C. albicans, we found that yeast cells adsorbed with either fresh serum or heated serum were strongly agglutinated when treated with anti-C3, slightly agglutinated with anti-IgG or anti-IgA and not agglutinated with anti-IgM. Serum either pretreated with anti-C3, anti-whole mouse, Zymosan at 37°C or EDTA did not support phagocytosis. However, C5-deficient serum and serum either treated with anti-IgG or EGTA was fully active. Serum treated to remove precursors of the alternative complement pathway supported an intermediate phagocytic activity (30%). Specific C. albicans antibody appears not to be responsible for any level of phagocytic activity observed in our test system because passage of fresh mouse serum through an affinity column containing C. albicans cell wall antigens did not alter the phagocytosis promoting activity of the serum. If serum was not added to the test system but if C. albicans adsorbed with fresh serum was used, 14% phagocytosis was observed. But if serum preabsorbed with C. albicans was used along with yeasts adsorbed with fresh serum, a high level of phagocytosis (72%) occurred. These data indicate that two serum factors will adsorb to C. albicans yeast cells and are required for optimum levels of phagocytosis. Other data are presented which indicate that the two factors are C3b and native C3. A third component, which is heat stable but not adsorbed by C. albicans is also required for maximum in vitro phagocytic activity.

INTRODUCTION

The importance of phagocytic cells in host resistance to infectious agents has been appreciated for many years. During the late 1800's, Eli Metchnikoff's theories concerning the role of phagocytic cells in resistance to infectious disease prompted much controversy among scientists (16,36). Up until that time most individuals believed that serum or humoral factors were the major defense mechanism of the host in protection against microbial invasion. The acceptance of both humoral and cellular aspects in resistance finally began to emerge during the early 1900's when Wright and Douglas in 1903 (16,69) demonstrated that both were important in the elimination of invading organisms. The term "opsonin" was introduced at this time and referred to factors present in serum which coat foreign particles and render them more readily ingestible by phagocytic cells (16,69). Many of the concepts of opsonins and opsonization put forth by these scientists still hold today and because of the importance of their work it would be worthwhile to summarize their findings. Wright and Douglas used human serum, human leukocytes, and staphylococci to determine the effect of serum on leukocyte phagocytosis of staphylococci. Briefly, some of their findings were: fresh serum is opsonic; opsonic activity of serum can be removed by either heating or by absorbing it with bacteria at 37°C; opsonins function by absorbing to the bacteria; and immune serum is more opsonic than

normal serum. The elegant studies performed by Wright and Douglas prompted much interest in the areas of opsonization and phagocytosis and today investigators are interested in identifying and chemically characterizing opsonins as well as opsonin receptors found on phagocytic cells.

Endocytosis is a cellular function that regulates the uptake of soluble (pinocytosis) and particulate (phagocytosis) substances (57), but because of the scope of this report only the phagocytic or ingestion process will be discussed. The process of phagocytosis, or the eating of particulate substances by cells, is carried out by professional and nonprofessional phagocytes. The primary function of professional phagocytes, such as polymorphonuclear leukocytes and macrophages, is ingestion of foreign particles. Although capable of phagocytosis, nonprofessional phagocytes such as fibroblasts, neurons and epithelial cells, do not phagocytose substances to the extent of professional phagocytes. Professional phagocytes have been used to study mechanisms of phagocytes and functions of membrane receptors. Although procedures for studying phagocytic functions vary among laboratories, most commonly the phagocyte is incubated with opsonized sheep red blood cells for a period of time, and then the cells are stained and the efficiency of phagocytosis is noted. Rabinovitch (50) separated the phagocytic process into two phases: attachment and

ingestion. Attachment of opsonized particles can be mediated by binding to Fc or C3 receptors found on macrophages and polymorphonuclear neutrophils (PMN) (1,13,26,31,32,56). Attachment does not, however, necessarily result in ingestion and may be dependent upon which receptor is involved. Two criteria used to distinguish between Fc and C3 receptors are sensitivity to trypsin and the requirement of divalent cations for attachment of opsonized particles to the appropriate receptor. The Fc receptor is resistant to digestion with trypsin and can bind IgG coated erythrocytes in the absence of divalent cations (18,20,21,25,35), whereas the C3 receptor is trypsin sensitive and requires divalent cations for attachment of C3 coated erythrocytes (31,32). The erythrocyte, opsonized with either immunoglobulins (IgG or IgM) or immunoglobulins and complement components (C3b or C3d), has been used to study the function and importance of Fc and C3 receptors in the attachment and ingestion of particles by phagocytic cells. In this system the Fc receptor for IgG mediates binding and ingestion of the IgG coated erythrocyte, whereas the C3 receptor mediates only binding of C3 coated erythrocytes to the phagocytic cells (31,32). Until recently it was thought that only the C3b fragment of C3 could mediate attachment to phagocytic cells, however, Ehlenberger and Nussenzweig (10) have shown that the C3d fragment of C3 can function as an opsonin if the phagocyte has the appropriate membrane receptor. They found

that human monocytes can bind both C3b and C3d opsonized particles, whereas human PMN lack the C3d receptor and can only bind C3b coated particles. On the other hand, Rabellino et al. (49) reported that most mouse neutrophils and less than half the mouse peritoneal macrophages bind C3d coated erythrocytes. Although no direct evidence has been presented for the existence of a membrane receptor for C5b on phagocytic cells, indirect evidence exists for the participation of C5 in the stimulation of phagocytosis by neutrophils (22,37,40).

A mechanism has been proposed by which a phagocyte internalizes an opsonized particle via receptor-particle interaction (14,15). In one study erythrocytes coated with IgG and C3b were allowed to adhere to macrophages via the C3b receptor. The uncombined C3b receptors were then destroyed with trypsin. Incubation of this macrophage-erythrocyte complex at 37°C did not result in uptake of the attached erythrocytes (14). In a more recent study, mouse lymphocytes bearing IgG were coated with the IgG fraction of anti-immunoglobulins (anti-immunoglobulin IgG) and either used directly in the phagocytic assay or allowed to cap at 37°C prior to use (15). It was found that lymphocytes diffusely coated with anti-immunoglobulin IgG became ingested by macrophages, whereas the lymphocytes that were allowed to cap became bound to the macrophage but were not ingested. From these studies it was concluded that: (1) mere binding of opsonized particles to the

membrane of a macrophage does not trigger ingestion; (2) opsonins must be present over the entire surface of the particle for ingestion to occur; (3) unbound receptors must be present on the surface of the phagocyte to where the particle is bound to the phagocytic cell membrane for ingestion to occur. This theory on ingestion of a particle by a phagocytic cell has been called the "zipper" mechanism of phagocytosis (14).

Immunoglobulins and components of complement have also been reported to be necessary for optimum phagocytosis of bacteria (11,17, 33,66,68) and fungi (7,30,37,40,59). Although opsonic requirements may differ from one particle to another, the mechanism of phagocytosis and the function of membrane receptors as shown with the erythrocyte-phagocyte system, has aided scientists in their attempt to identify and functionally characterize opsonins required for ingestion of various particles.

Recent evidence suggests that the serum requirements for opsonization of bacteria vary not only among different genera and species of bacteria, but also among strains of a bacterial species as well. Serum IgG and heat-labile opsonins have both been shown to induce phagocytosis of Staphylococcus aureus (17,68). Verhoef et al. (66) reported that several strains of S. aureus and Staphylococcus epidermidis could be opsonized with specific antibody and components of either

the classical or alternative complement pathway. They also showed a heterogeneity in opsonic requirements for the strains of S. aureus and S. epidermidis used in their study. Giebink et al. (11) reported variability in opsonic requirements of four strains of Streptococcus pneumoniae. It has been shown that Escherichia coli requires heat-labile serum factors, presumably C3, for optimum phagocytosis (17). McLean et al. (33) have presented evidence to suggest that serum factor B, a precursor of the alternative complement pathway, is required for optimum phagocytosis of E. coli. In this study (33), sera deficient in factor B supported an intermediate level of phagocytosis when compared to normal serum. The normal opsonic function of these sera, deficient in factor B and deficient in opsonization, could be restored with the addition of purified factor B.

Complement components and immunoglobulins seem to be potent opsonins for bacteria, however, little has been done to characterize the function of the Fc and C3 receptors in bacterial phagocytosis. Verhoef et al. (67) have recently attempted to characterize Fc and complement receptors on PMN. Their data suggests that both Fc and C3 receptors participate in the attachment and ingestion phases of phagocytosis. Staphylococci opsonized in the presence of immunoglobulin and complement are primarily attached to the phagocyte and ingested via the complement receptor. Somewhat different functions

have been assigned to the Fc and C3 receptors on mononuclear phagocytes, when sheep erythrocytes are used as the opsonized particle (31,32). In this system the Fc receptor for IgG mediates both binding and ingestions of the particle, whereas the complement receptors mediate only binding of the particle to the phagocyte. In contrast to the phagocyte-erythrocyte system, the C3 receptor on PMN promotes attachment and ingestion of staphylococci.

Mechanisms of host resistance to fungal infections are controversial at this time. Several studies indicate that cell-mediated immunity (CMI) is an important aspect of this resistance (2,8,23,26,48,61,62). Stobo et al. (61) were able to demonstrate that patients with various fungal diseases had a T-cell dysfunction in which a population of T-cells was suppressing potentially active cells. It has been proposed that patients with chronic mucocutaneous candidiasis have some defect in T-cell function (23,63). Some patients with this syndrome show high agglutinin titers to candida antigen but are unable to mount delayed type hypersensitivity reactions to purified protein derivative, dinitrochlorobenzene, mumps antigen, and candida antigen (23,63). Takeya et al. (63) concluded that T-cells from these patients were able to act as helper cells in antibody production, but have a dysfunction in their ability to develop cellular immunity. Miyake et al. (39) have suggested that protection against candida infection

involves both nonimmune phagocytic cells and cell-mediated immunity. They propose that nonimmune phagocytosis is important in early stages of infection, while T cell-mediated immunity is required at the later stages of the infection.

Humoral immunity has been thought not to be of primary importance in resistance to candidiasis. Recently, however, it was reported that humoral immunity contributes to the protection of the host against experimental deep-seated candidiasis (45). This study showed that the transfer of immune serum offered protection against candidal infection while immune cells did not.

Although data indicate that CMI and humoral immunity may be important mechanisms of host resistance to fungal disease, phagocytic cells have drawn much attention over the past years as to their role in host resistance to these types of diseases. It has been proposed that phagocytic cells may be important in resistance to fungal disease in humans (9,28,30,41,60,65) and experimental animals (3,5,19,39,52). Recently, it was demonstrated by Cutler (5) and Rogers et al. (52) that congenitally thymic deficient (nude) mice are more resistant to an acute infection with Candida albicans than are their normal littermates. This increased resistance was thought to be due to phagocytic cells. BCG immunized mice, however, were no more resistant to experimental candidiasis than normal mice (53). In this latter study the

authors indicate that PMN may be important in resistance to experimental candidiasis, but present no data to support this claim. In a recent study by Corbel et al (3) it was shown that athymic mice were no more susceptible to experimental mucormycosis than their normal littermates. The authors suggest that their resistance is possibly due to an enhanced nonspecific cellular response. Staples et al. (60) reported about a young girl with disseminated candidiasis whose immunological functions seemed normal, in that she had detectable anti-candida antibodies; she developed delayed type hypersensitivity to candida antigen; she had normal levels of immunoglobulin and complement; and her leukocytes phagocytosed bacteria and C. albicans. However, although her leukocytes ingested C. albicans, they failed to kill the fungus. Her leukocytes, however, were bactericidal for various bacteria and fungicidal for another Candida species.

The above studies suggest that phagocytic cells play a role in host resistance to fungal infections. Because of this, an understanding of the requirements for ingestion of fungi by phagocytic cells is needed. Howard (19), Tacker et al. (62), Mitchell and Friedman (38), and Lehrer and Cline (27) have shown that fresh serum is required for optimum phagocytosis of Histoplasma capsulatum, Cryptococcus neoformans and C. albicans. Because heat-labile serum factors are needed for the phagocytosis of C. neoformans (6,38) and

C. albicans (27,30,55), it has been assumed that components of complement are important. Diamond et al. (7) suggest that both the classical and alternative pathways of complement activation are required for optimum opsonization of C. neoformans. Morelli and Rosenberg (40) imply that complement component C5 is required for optimum leukocyte phagocytosis of C. albicans. In their test system they found that mouse leukocytes, obtained from normal or C5-deficient mice, incubated with C. albicans and normal mouse serum showed a higher degree of phagocytosis at 10 minutes of incubation than cells incubated in C5-deficient serum. However, by 30 minutes incubation, there was no significant difference in the amount of phagocytosis. These authors also report that phagocytosis by mouse peritoneal cells was the same whether normal or C5-deficient serum was used. More recently it has been proposed by Solomkim et al. (59) that several serum factors may participate in the opsonization of C. albicans. They found that serum treated with ethyleneglycoltetraacetic acid (EGTA), a chelator of calcium ions and an inhibitor of the classical pathway of complement activation, was less opsonic than normal serum, but more opsonic than heat-activated serum. Heat-inactivated serum, however, supported higher levels of phagocytosis than no serum at all. They proposed that C. albicans is primarily opsonized via the classical pathway of complement activation, but the alternative pathway and heat-stable serum factors,

presumably immunoglobulin, can also contribute to the opsonic process.

The primary defense mechanism of the host in protecting itself from infection by C. albicans is controversial at this time. Scientists have presented data which suggest that either CMI, humoral immunity, phagocytic cells, or a combination of these defense mechanisms function in host resistance to candidiasis. Because phagocytic cells have been implicated in host defense to candidiasis, an understanding of the requirements for ingestion of C. albicans is desirable. The purpose of this project was to identify serum factors that contribute to the phagocytosis of C. albicans by mouse peritoneal macrophages, and to determine to what extent these factors contribute to ingestion of the fungus.

MATERIALS AND METHODS

Source of experimental animals. BALB/c mice, originally obtained from Jackson Laboratories (Bar Harbor, ME), C5-deficient B10.D2/OSn mice, and C5-deficient DBA/2 mice (Jackson Laboratories) were raised and maintained in our animal rooms. All feed, bedding, cages and water bottles were sterilized prior to use and only acidified water was used (34). 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 animals had neither detectable candida agglutinins nor precipitins.

Preparation of macrophages. Peritoneal exudate cells were obtained from 6 week to 4 month old male or female BALB/c mice. The animals were killed by cervical dislocation, the skin over the abdominal region was reflected, and a small incision was made into the peritoneal cavity. Using a Pasteur pipette, the cavity was washed with 2 to 5 ml of Hank's Balanced Salts Solution (HBSS) + 0.002M Ethylenediamine-tetraacetic acid (EDTA). The washings were centrifuged at 200 x g for 10 min. The cells were washed twice with HBSS (without EDTA) and once with medium 199 (M199) (Gibco, Grand Island, NY). The cells were counted in a hemocytometer and resuspended to 2×10^6 cells/ml in M199. Viability was determined by trypan blue exclusion and cell types were determined by microscopic examination of Giemsa

stained smears. In all experiments, viability of the peritoneal cells exceeded 90% and over 85% were mononuclear cells.

Yeasts and growth condition. Candida albicans strain 9938 (Medical Mycology Unit, Tulane University), C. albicans strain Sweenie (obtained from Dr. T.G. Mitchell, Duke University), C. albicans strain 69 (a gift from Dr. A. Weeks, Old Dominion University), Torulopsis glabrata (Montana State University) and Baker's yeast were grown aerobically in 2.0% glucose-0.3% yeast extract-1.0% peptone broth. The cultures were rotated at 180 rpm (Gyrotatory Incubator - Shaker, New Brunswick, NJ) for 24-72 hrs at 37°C. Organisms were harvested by centrifugation, washed three times in saline, and resuspended in M199 to a concentration of 4×10^6 yeast cells/ml. Viability was greater than 95% as determined by methylene blue (27). Candida albicans strain 9938 was used in all experiments unless stated otherwise.

Serum source. Male or female BALB/c, C5-deficient B10.D2/OSn and C5-deficient DBA/2 mice were lightly anesthetized with ether and bled from the orbital plexus. The blood was allowed to clot at 0-4°C for 30-60 min and then centrifuged at 460 x g for 30 min at 4°C. The serum was removed, kept in an ice-water bath (0-4°C) and used within 4 hr of collection. Serum collected in this manner is referred to as fresh mouse serum.

Antisera source and preparation. Anti-whole mouse serum, anti-mouse IgG, anti-mouse IgM, and anti-mouse IgA were obtained commercially (Meloy, Springfield, VA). Anti-mouse C3 was either purchased from Cappel Laboratories (Cochrainville, PA) or prepared by us in the following manner. Precipitin bands which formed in agar double-diffusion plates from the reaction of C3 (using fresh mouse serum as the source of C3) with anti-C3 were cut from the agar and soaked in 0.01M phosphate buffered saline (pH 7.2) at 4°C for 10 days. During this time the buffer was frequently changed to remove non-precipitated protein. The washed precipitin bands were homogenized with a glass tissue grinder and emulsified with either complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA). Rabbits were given two injections of precipitin-CFA 3 days apart followed by two booster injections of precipitate-IFA one week apart. Ten days after the last booster the rabbits were bled by cardiac puncture and the sera were obtained. Before use all antisera were absorbed 3-5 times with C. albicans and Zymosan (Nutritional Biochemical Corporation, Cleveland, OH). The various antisera received from commercial sources or prepared by us were used only if they produced a single precipitin band in double diffusion agarose plates (Ouchterlony) when tested against fresh mouse serum. When tested in adjacent wells against fresh serum, none of the antisera developed lines of identity or partial identity.

Antisera against C. albicans strain 9938 were produced in rabbits by sensitizing and boosting the animals with heat killed whole yeast cells emulsified in either CFA or IFA, respectively, at a concentration of 0.5 mg yeast cells (dry weight) per 1 ml emulsion. The animals were sensitized by inoculating 1 ml of the emulsion into each of four different subcutaneous locations. Three days and six days later the rabbits were reinjected using a similar injection protocol. Seven days after the second booster, the animals were again boosted and the sera were collected 10 days after the third booster. Sera from animals that gave a tube agglutinin titer of 512 or greater were pooled and stored at -20°C until use.

Immunoelectrophoresis of antisera. 0.75% electrophoretic agar (MCI Biomedical Rockland, ME) was melted in either 0.1 M Tris (hydroxymethyl aminomethane) buffer or barbitol buffer, pH 8.6. Appropriate wells and troughs were prepared in agar on glass slides using a Gel Punch (Gelman Inst. Co., Ann Arbor, MI); sera or antisera were placed in appropriate wells and electrophoresed for 2 hrs at 15 mamps (150-250 volts). Appropriate antisera were added to the troughs and the bands were allowed to develop at room temperature for 18-48 hrs.

Double diffusion in agar. Double diffusion slides (Ouchterlony) were prepared by adding 5 ml of melted 0.75% agarose (MCI Biomedical, Rockland, ME) in saline to a glass slide. After adding the appropriate

antigens and antisera to the wells, the slides were placed in a humidified chamber and the bands were allowed to develop at room temperature for 12 to 48 hrs.

Adsorption of yeast cells with mouse sera. 4.0×10^6 C. albicans were suspended in 0.5 ml of appropriate serum and incubated at 37°C for 20 min. The cells were pelleted by centrifugation and the absorbed serum was removed. The fungal cells (adsorbed yeasts) were then washed in 6 ml of saline and kept at $0-4^\circ\text{C}$ until use.

Adsorption of yeast cells with rabbit anti-C. albicans. 4×10^6 washed C. albicans were suspended in 1 ml of M199 containing just enough rabbit anti-C. albicans to cause slight agglutination of the yeast cells. The suspension was incubated 15 min at 37°C , the fungal cells were then centrifuged and washed in 6 ml of saline and kept at $0-4^\circ\text{C}$ until use. Yeast cells adsorbed in this manner were strongly agglutinated when added to a drop of goat anti-rabbit gamma globulin (Colorado Serum Co., Denver, CO) whereas unadsorbed yeast cells did not agglutinate in this reagent.

Observation of phagocytosis using scanning electron microscopy. After various times of incubation, cover glasses containing phagocytic cells and yeast cells were flooded with 2.5% glutaraldehyde in PBS at room temperature for 30 min. After fixation the cover glasses were rinsed with PBS and postfixed for 30 min with 1% osmium tetroxide in

PBS. The cover glasses were rinsed again with PBS and the cells were dehydrated by rinsing the cover glasses in a graded series of acetone. The cells were sputter-coated with gold and examined in a Super Mini-SEM II (International Scientific Instruments, Inc., Mountain View, CA).

Treatment of Serum. To determine which serum factors are required for the phagocytosis of C. albicans, fresh mouse serum was treated in the following ways: 1. Heat labile serum components were inactivated by incubating serum at either 56°C for 30 min or at 50°C for 30 min. 2. Serum components which adsorb to C. albicans were removed from the serum by five absorptions with yeast cells at a ratio of 1 volume of serum to 0.1 volume of packed cells. The yeast cell-serum combination was allowed to incubate at 37°C for 15 min during each absorption. 3. Fresh serum was treated with 1 mg Zymosan/ml of serum by either absorbing the serum two times with the Zymosan at 37°C for 20 min (43) or by absorbing the serum once at 17°C for 60 min (47). 4. Serum was treated with either EDTA or ethyleneglycol tetraacetic acid (EGTA) (Sigma Chem. Co., St. Louis, MO) to chelate Ca^{++} and Mg^{++} ions or Ca^{++} ions, respectively (43). Enough fresh serum was added to either HBSS (Ca^{++} and Mg^{++} free) + 0.01M EDTA or HBSS (Ca^{++} and Mg^{++} free) + 0.01M EGTA + 0.01M MgCl_2 to give a concentration of 5% serum and then incubated at 37°C for 15 min. After

incubation, yeast cells were suspended in one of the above media and used in the phagocytic test system. Because of chelating properties, treatment of serum with EDTA will prevent activation of the classical and alternative pathways of complement activation, while treatment with EGTA will prevent only activation of the classical pathway (43). Appropriate controls were run to determine the effect of EDTA on macrophages. 5. Fresh mouse serum was passed through an affinity column containing candida antigen to remove specific C. albicans antibody. Serum passed through the column was used directly or heat-inactivated prior to use in the phagocytic assay.

Preparation of soluble antigen. The soluble antigen was recovered from the aqueous phase of a hot phenol extract (PE) of whole cells of C. albicans prepared as reported previously (6).

Removal of anti-C. albicans antibodies from fresh mouse serum. An affinity column was prepared by coupling the antigen (PE) to Sepharose 6B (Pharmacia, Piscataway, NJ) as previously reported (A.H. Poor and J.E. Cutler, manuscript submitted for publication). After the coupling procedure was completed the beads were washed with distilled water, packed in two glass columns (1.5cm x 5.0cm) and equilibrated with M199 at 4°C. A control column (1.5cm x 5.0cm) was prepared with activated Sepharose 6B without PE. The efficiency of the affinity column for removing anti-C. albicans antibody was tested by passing

3.5ml of immune serum through the column and determining the agglutinin titer of the serum before and after column treatment (See Results). 1 ml of fresh normal mouse serum was then washed either through the Sepharose 6B-PE affinity column with M199 to remove anti-C. albicans antibodies or through the control column. The serum was collected and either used directly or heat inactivated at 56°C for 30 min prior to use.

Phagocytic assay. 0.5 ml of the peritoneal macrophage suspension (2×10^6 cells/ml in M199), with or without mouse serum, was added to each 22 mm x 22 mm cover glass (VWR Scientific, Seattle WA) and incubated in a humidified chamber at 37°C for 1 hr. The non-adherent cell population was then removed by rinsing the cover glasses with 2 ml of HBSS. 0.5 ml of a suspension of C. albicans containing 4×10^6 yeast cells/ml in M199 with or without mouse serum was added to the cover glasses containing the macrophages and incubated at 37°C for 1 hr. After various times of incubation the cover glasses were washed with 2 ml of HBSS to remove yeasts not attached to nor ingested by macrophages. The cover glasses were either processed for scanning electron microscopy (see above) or, more commonly, 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 particles.

All tests were run on at least two different occasions.

RESULTS

Phagocytic activity remained essentially constant over a 2.5%-50% range of serum concentration but decreased sharply when the serum concentration was below 2.5% (Fig. 1). We subsequently used a 5% serum concentration in our test system.

The optimum incubation time was determined by examining Giemsa stained monolayers of macrophages and yeasts on cover glasses after various times of incubation (Fig. 2) and by viewing appropriately fixed cells on cover glasses using a scanning electron microscope (Figs. 3,4, and 5).

From these observations we determined that by 15 min of incubation most of the yeast cells were adsorbed to the cell membrane of the macrophages but very little ingestion had occurred (Fig. 4). By 60 min incubation most of the yeast cells had become ingested (Fig. 5). A one-hour incubation of yeasts and macrophages was subsequently used.

The effect of various treatments of serum on the phagocytosis of *Candida albicans*. Fresh mouse serum supported the greatest phagocytic activity when compared with mouse serum that had been either preheated at 56°C or absorbed with *C. albicans* (Table 1). Three distinct levels of phagocytic activity were detected. The highest level of activity, when fresh serum was used, will be referred to as optimum phagocytic activity. An intermediate level of activity occurred when serum was preheated at 56°C, and a low level of activity occurred when serum was either omitted from the test system or serum was preabsorbed with

Fig. 1. The effect of increasing amounts of serum on the phagocytosis of C. albicans by mouse peritoneal macrophages.

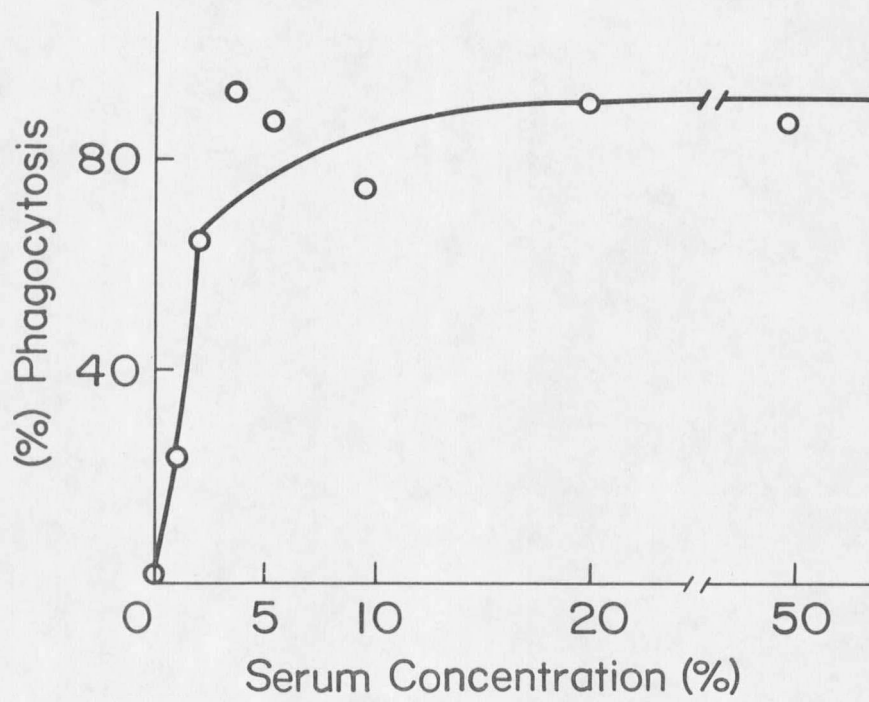


Fig. 2. The effect of incubation time at 37°C on the phagocytosis of C. albicans by mouse peritoneal macrophages. Open bars represent the results obtained when fresh serum was used; the closed bars represent data obtained when heated (56°C, 30 min) serum was used.

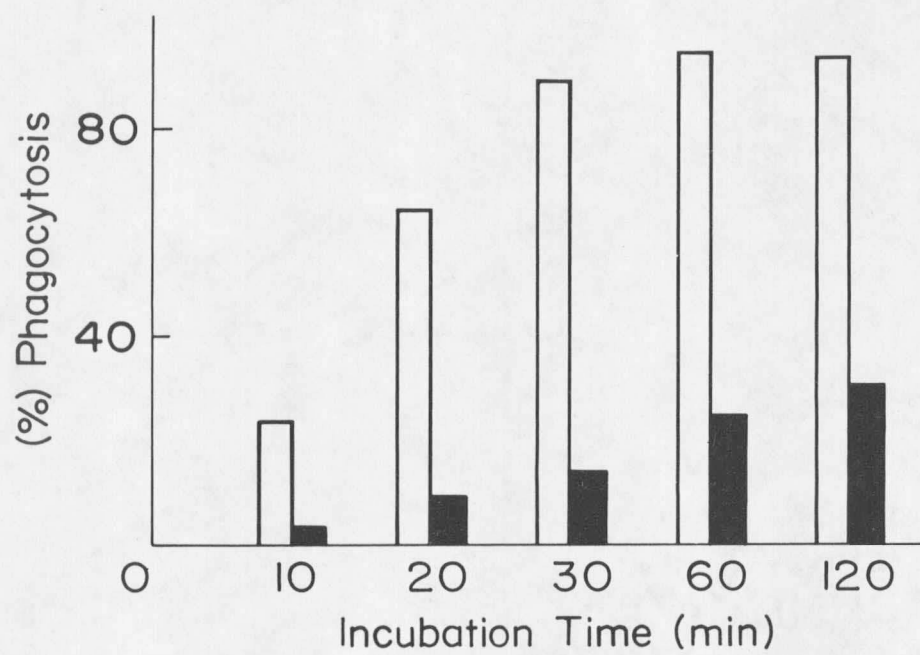


Fig. 3. Scanning electron micrograph of mouse peritoneal cells which were allowed to adhere to the cover glass at 37°C for 10 min before fixation (bar = 10 μ m)

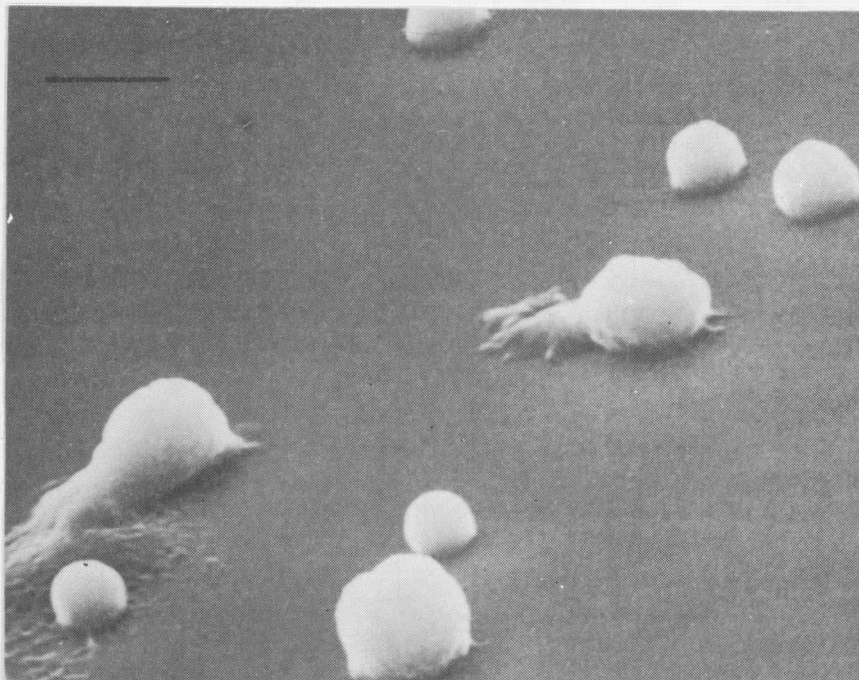


Fig. 4. Scanning electron micrographs of mouse peritoneal cells that were allowed to interact with C. albicans in the presence of 5% fresh mouse serum for 15 min before fixation (A, bar = 5 μm ; B, bar = 8 μm).

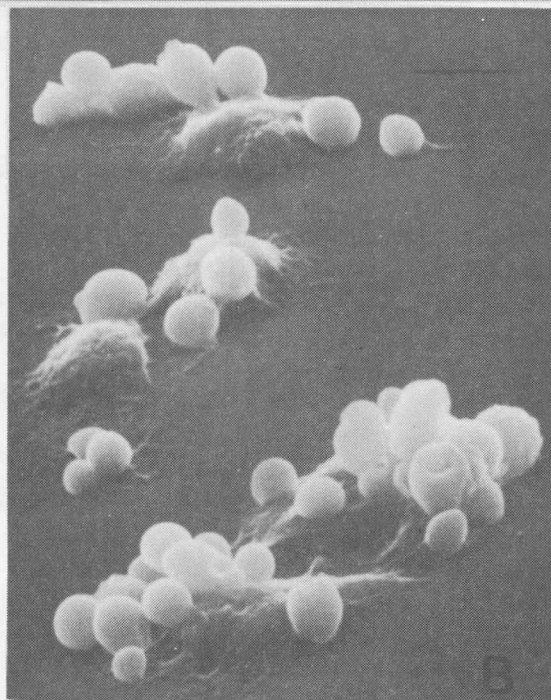
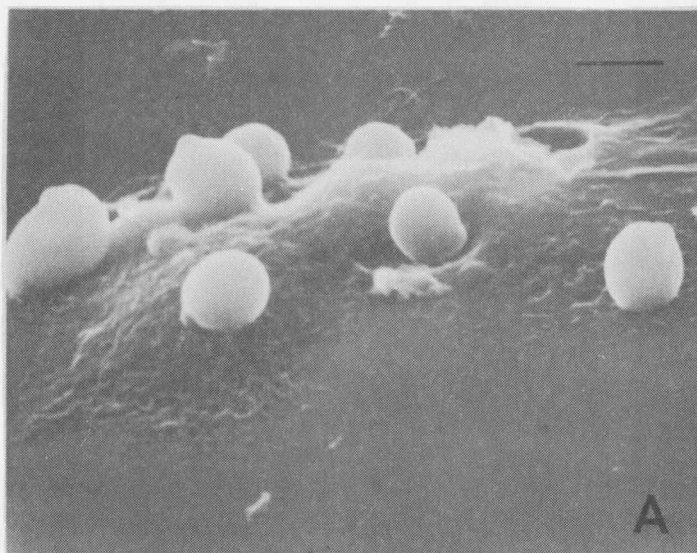


Fig. 5. Scanning Electron micrographs of mouse peritoneal cells that were allowed to interact with C. albicans in the presence of 5% fresh serum for 60 min before fixation (A, bar = 2 μm ; B, bar = 5 μm).

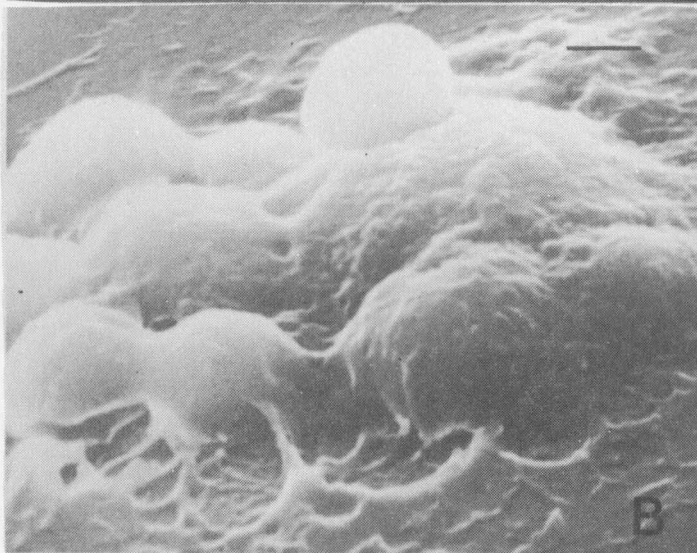
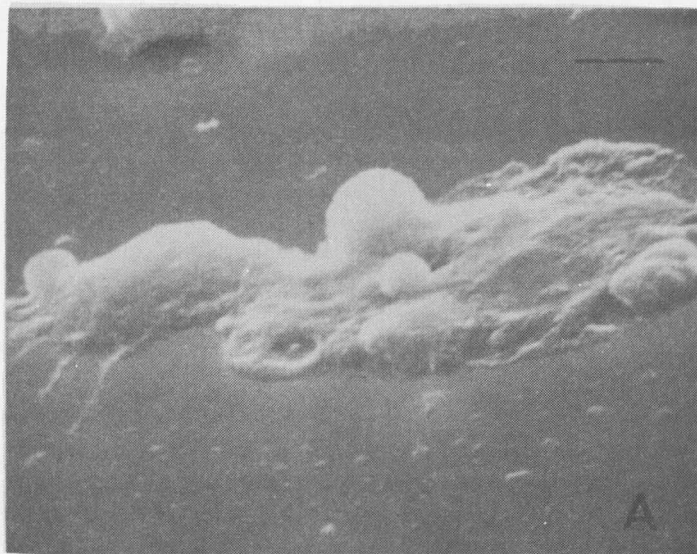


TABLE 1. PHAGOCYTOSIS OF CANDIDA ALBICANS BY MOUSE PERITONEAL MACROPHAGES IN THE PRESENCE OF EITHER FRESH SERUM, HEAT-INACTIVATED SERUM, SERUM ABSORBED WITH C. ALBICANS, OR IN THE ABSENCE OF SERUM FROM THE IN VITRO TEST SYSTEM.

CONDITION OF SERUM	% PHAGOCYTOSIS
FRESH	85.1 ± ¹ 6.7
HEATED (56°C, 30 MIN)	22.0 ± 10.1
ABSORBED WITH <u>C. ALBICANS</u>	4.8 ± 3.3
SERUM NOT ADDED	2.5 ± 1.6

¹ STANDARD DEVIATION OF THE MEAN OF AT LEAST 5 SEPARATE EXPERIMENTS RUN IN DUPLICATE EACH TIME.

C. albicans. Fresh mouse serum also supported a level of phagocytosis greater than that supported by heat-inactivated serum when other strains of C. albicans and yeasts or yeast-like fungi were used in the phagocytosis test (Table 2).

Serum factors which adsorb to C. albicans. Because the phagocytosis promoting activity of mouse serum was lost when the serum was preabsorbed with C. albicans, we attempted to identify some of the serum components which adsorb to the yeast particles. C. albicans which had been adsorbed with either fresh mouse serum or serum that was preheated at 56°C was tested for agglutinability in the presence of sera containing antibodies to various mouse serum proteins (Table 3). Anti-mouse C3 produced the strongest agglutination reactions when tested against C. albicans adsorbed with either fresh mouse serum or serum preheated at 56°C. Some agglutination also occurred upon mixing adsorbed cells with anti-mouse IgA and anti-mouse IgG, but agglutination was not apparent when anti-mouse IgM was used.

Serum collected from rabbits which were immunized with adsorbed yeasts produced a single precipitin band when tested against mouse serum; this band formed a line of identity with a precipitin band which developed due to the interaction of anti-C3 and mouse serum.

These data indicate that at least three mouse serum components, C3, IgG, and IgA, will adsorb to C. albicans. Of these three, comple-

TABLE 2. PHAGOCYTOSIS OF VARIOUS YEASTS AND YEAST-LIKE FUNGI IN THE PRESENCE OF EITHER FRESH MOUSE SERUM OR HEATED MOUSE SERUM (56°C FOR 30 MIN).

TYPE OF YEAST	% PHAGOCYTOSIS	
	FRESH SERUM	HEATED SERUM
<u>CANDIDA ALBICANS</u> STRAIN 9938	85.1 ± ¹ 6.7	22.0 ± 10.7
<u>CANDIDA ALBICANS</u> STRAIN SWEENIE	91.1 ± 4.6	41.0 ± 12.3
<u>CANDIDA ALBICANS</u> STRAIN 69	92.0 ± 3.7	24.3 ± 8.2
<u>TORULOPSIS GLABRATA</u>	88.4 ± 4.6	38.6 ± 13.9
<u>SACCHAROMYCES CEREVISIAE</u>	92.8 ± 7.1	26.5 ± 9.7

¹STANDARD DEVIATION OF THE MEAN OF AT LEAST TWO SEPARATE EXPERIMENTS RUN IN DUPLICATE EACH TIME.

TABLE 3. SERUM COMPONENTS WHICH ADSORB TO C. ALBICANS DETECTED BY AGGLUTINATION USING VARIOUS ANTISERA.

SERUM USED FOR ADSORPTION	AGGLUTINATION OF ADSORBED <u>C. ALBICANS</u> BY SPECIFIC ANTISERA				
	ANTI-WHOLE MOUSE	ANTI- IGM	ANTI- IGG	ANTI- IGA	ANTI- C3
FRESH	4+	¹ NA	2+	2+	4+
HEATED (56°C, 30 MIN)	3+	NA	1+	1+	3+
ABSORBED WITH <u>C. ALBICANS</u>	NA	NA	NA	NA	NA
NONE	NA	NA	NA	NA	NA

¹NA = NO AGGLUTINATION

ment component C3 seems to be most readily detectable on the adsorbed yeast cells.

Identification of opsonic factors. In an attempt to identify which of the serum factors function opsonically, components of mouse serum were selectively removed and the resulting sera were tested for phagocytosis promoting activity (Table 4). Low phagocytic activity occurred if the mouse serum used in the test was pretreated with either anti-whole mouse serum or anti-mouse C3. Serum also lost the ability to promote phagocytosis if it was either preabsorbed with Zymosan at 37°C or treated with EDTA. Control experiments showed that EDTA does not adversely affect macrophage function (see below). Serum pretreated with either anti-IgG or EGTA supported optimum phagocytosis. An intermediate phagocytic activity was observed if the serum was either preabsorbed with Zymosan at 17°C, or if the serum was heated at 50°C for 30 min before use in the test. Mouse serum deficient in complement component C5 (B10.D2/Osn or DBA/2) supported optimum phagocytosis. If these sera were preheated at 56°C for 30 min an intermediate level of phagocytic activity was observed and a low (<7%) phagocytic activity was obtained if the sera were preabsorbed with C. albicans at 37°C prior to use. The results obtained with C5-deficient serum from either B10.D2/Osn or from DBA/2 were the same whether BALB/c, B10.D2/Osn or DBA/2 macrophages were used.

TABLE 4. THE PHAGOCYTOSIS PROMOTING ACTIVITY OF MOUSE SERUM AFTER VARIOUS TREATMENTS.

SERUM TREATMENT	% PHAGOCYTOSIS SUPPORTED BY THE TREATED SERA
CONTROL (FRESH SERUM)	85.1 ± ¹ 6.7
ANTI-WHOLE MOUSE	5.9 ± 1.7
ANTI-C3 (AT 37°C OR 40°C)	5.3 ± 1.8
ANTI-IGG (40°C)	87.8 ± 2.0
HEATED AT 50°C, 30 MIN	48.8 ± 13.7
ZYMOSAN ABSORBED AT 37°C	5.3 ± 2.0
ZYMOSAN ABSORBED AT 17°C	23.6 ± 8.8
EDTA	9.4 ± 8.5
EGTA	76.0 ± 8.0
² C5-DEFICIENT (B10.D2/OSN)	85.0 ± 1.5

¹STANDARD DEVIATION OF THE MEAN OF AT LEAST THREE SEPARATE EXPERIMENTS RUN IN DUPLICATE EACH TIME.

²THIS SERUM IS NOT TREATED IN ANY WAY, BUT IS SERUM OBTAINED FROM MICE GENETICALLY DEFICIENT IN C5. ALSO, SERUM OBTAINED FROM DBA/2 MICE, WHICH ARE ALSO GENETICALLY DEFICIENT IN C5, SUPPORTED SIMILAR PHAGOCYTTIC ACTIVITY.

These data indicate that there are at least two serum components required for phagocytosis; one which supports an optimum phagocytic activity and one which supports an intermediate level of phagocytosis.

Evidence that EDTA does not adversely affect macrophage function.

If macrophages were allowed to adhere to cover glasses in the presence of 0.01M EDTA, then washed and presented with yeasts in 5% fresh mouse serum, optimum phagocytosis occurred (Table 5). If EDTA was also included in the yeast-serum suspension, a low (<5%) phagocytic activity occurred. Finally, if the macrophages were allowed to settle in the presence of EDTA, then were presented with yeasts in EDTA, incubated for 1 hr, washed and presented with yeasts in 5% fresh serum without EDTA, a high level of phagocytosis activity was restored (Table 5).

Evidence for a third serum factor required for an optimum level of phagocytosis. The following series of experiments were designed to determine if serum constituents were required during macrophage adherence to cover glasses, during presentation of the yeasts to the macrophages, or during both of these periods of incubation. The data obtained indicate that serum factors must be present during both time periods (Table 6). However, mouse serum preabsorbed with C. albicans may substitute for fresh serum during the time interval in which macrophages are settling onto the cover glasses. Also, optimum phagocytosis occurred in the test system when serum preabsorbed with C. albicans

TABLE 5. EVIDENCE THAT EDTA DOES NOT ADVERSELY AFFECT PHAGOCYTIC ACTIVITY OF MACROPHAGES

TYPE OF SERUM ADDED TO MACROPHAGES	TYPE OF SERUM ADDED WITH YEASTS	CONDITION OF YEASTS	% PHAGOCYTOSIS
FRESH	FRESH	¹ NORMAL	85.1 ± ² 6.7
FRESH + EDTA	FRESH	NORMAL	76.7 ± 4.7
FRESH	FRESH + EDTA	NORMAL	4.2 ± 1.7
FRESH + EDTA	³ FRESH + EDTA, THEN FRESH	NORMAL	66.1 ± 7.2

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¹YEAST PHASE ORGANISMS WERE WASHED THREE TIMES IN SALINE AND USED IN TEST

²STANDARD DEVIATION OF THE MEAN OF AT LEAST TWO SEPARATE EXPERIMENTS RUN IN DUPLICATE EACH TIME

³MACROPHAGES WERE FIRST PRESENTED WITH YEASTS IN 5% SERUM + EDTA AND INCUBATED AT 37°C FOR 1 HR; THE MACROPHAGES WERE THEN WASHED WITH HBSS, PRESENTED WITH YEASTS IN 5% SERUM WITHOUT EDTA, INCUBATED FOR AN ADDITIONAL 1 HR AND PERCENT PHAGOCYTOSIS WAS DETERMINED

TABLE 6. EVIDENCE FOR A THIRD SERUM FACTOR WHICH IS REQUIRED BY MOUSE PERITONEAL MACROPHAGES FOR OPTIMUM IN VITRO UPTAKE OF C. ALBICANS, BUT IS NOT ABSORBABLE BY C. ALBICANS.¹

TYPE OF SERUM ADDED TO MACROPHAGES	TYPE OF SERUM ADDED WITH YEASTS	CONDITION OF YEASTS	% PHAGOCYTOSIS
FRESH	FRESH	² NORMAL	85.1 ± ⁴ 6.7
NONE	NONE	NORMAL	2.5 ± 1.6
NONE	FRESH	NORMAL	35.9 ± 11.4
FRESH	NONE	NORMAL	7.4 ± 1.6
ABSORBED WITH <u>C. ALBICANS</u>	ABSORBED WITH <u>C. ALBICANS</u>	NORMAL	4.8 ± 3.3
ABSORBED WITH <u>C. ALBICANS</u>	FRESH	NORMAL	77.5 ± 5.1
FRESH	ABSORBED WITH <u>C. ALBICANS</u>	NORMAL	13.8 ± 8.6
NONE	NONE	³ ADSORBED WITH FRESH SERUM	13.4 ± 2.5
ABSORBED WITH <u>C. ALBICANS</u>	ABSORBED WITH <u>C. ALBICANS</u>	ADSORBED WITH FRESH SERUM	81.5 ± 7.5
NONE	ABSORBED WITH <u>C. ALBICANS</u>	ADSORBED WITH FRESH SERUM	13.0 ± 3.4

¹MACROPHAGES WERE ALLOWED TO ADHERE TO COVER GLASSES EITHER IN THE PRESENCE OF FRESH MOUSE SERUM, MOUSE SERUM ABSORBED WITH C. ALBICANS, OR IN THE ABSENCE OF SERUM. AFTER ADHERENCE TO COVER GLASSES, THE MACROPHAGES WERE RINSED AND THE YEASTS WERE ADDED IN THE PRESENCE OR ABSENCE OF SERUM COMPONENTS.

²YEAST PHASE ORGANISMS WERE WASHED THREE TIMES IN SALINE AND USED IN THE TEST.

³WASHED YEAST PHASE ORGANISMS WERE INCUBATED IN FRESH MOUSE SERUM AT 37°C FOR 20 MIN, THEN WASHED ONCE IN 6 ML OF SALINE AND USED IN THE TEST.

⁴STANDARD DEVIATION OF THE MEAN OF AT LEAST THREE SEPARATE EXPERIMENTS RUN IN DUPLICATE EACH TIME.

was used along with *C. albicans* adsorbed with fresh serum.

Evidence that *C. albicans* specific antibody is not responsible for the intermediate phagocytic activity. An intermediate phagocytic activity (31.5%) occurred if yeasts used in the test were preadsorbed with anti-*C. albicans*, but preabsorption of the immune serum with Zymosan did not remove this level of opsonic activity (Table 7). The opsonic factor(s) of immune serum was removed by absorption with *C. albicans* at 4°C whereas the phagocytosis promoting factors of either fresh mouse serum or heated mouse serum were not removed by absorption with *C. albicans* at 4°C (Table 7). Also, an intermediate activity (24.5%) occurred if the yeasts used in the test were preabsorbed with immune serum at 4°C, whereas a low level occurred (10.7%) if the yeasts were preabsorbed with fresh mouse serum at this temperature (Table 7).

One passage of rabbit serum containing antibodies against whole cells of *C. albicans* (agglutinin titer of 128) through the Sepharose 6B-PE affinity column removed approximately 93% of the agglutinins and 50% of the antibody activity could be recovered by eluting the column with 50ml of 0.5M glucose followed by 50ml of 0.5M mannose (A.H. Poor and J.E. Cutler, submitted for publication). Passage of fresh mouse serum through the affinity column did not, however, decrease the level of phagocytosis (Table 8). Also heat inactivation of mouse serum passed through the affinity column still supported intermediate phagocytic activity.

TABLE 7. THE EFFECT OF ANTIBODY ON THE PHAGOCYTOSIS OF CANDIDA ALBICANS

TYPE OF SERUM ADDED TO MACROPHAGES	TYPE OF SERUM ADDED WITH YEASTS	CONDITION OF YEASTS	% PHAGOCYTOSIS
ABSORBED WITH <u>C. ALBICANS</u> AT 37°C	ABSORBED WITH <u>C. ALBICANS</u> AT 37°C	ADSORBED WITH ANTI- <u>C. ALBICANS</u>	31.5 ± 10.5
ABSORBED WITH <u>C. ALBICANS</u> AT 37°C	ABSORBED WITH <u>C. ALBICANS</u> AT 37°C	² ADSORBED WITH ZYMOSAN TREATED ANTI- <u>C. ALBICANS</u>	33.3 ± 13.8
³ ANTI- <u>C. ALBICANS</u> ABSORBED WITH <u>C. ALBICANS</u> AT 4°C	ANTI- <u>C. ALBICANS</u> ABSORBED WITH <u>C. ALBICANS</u> AT 4°C	NORMAL	8.8 ± 4.1
⁴ MOUSE SERUM ABSORBED WITH <u>C. ALBICANS</u> AT 4°C	MOUSE SERUM ABSORBED WITH <u>C. ALBICANS</u> AT 4°C	NORMAL	78.6 ± 9.9
⁵ MOUSE SERUM ABSORBED AT 4°C, THEN HEAT INACTIVATED	MOUSE SERUM ABSORBED AT 4°C, THEN HEAT INACTIVATED	NORMAL	28.3 ± 4.9
ABSORBED WITH <u>C. ALBICANS</u> AT 37°C	ABSORBED WITH <u>C. ALBICANS</u> AT 37°C	ADSORBED WITH ANTI- <u>C. ALBICANS</u> AT 4°C	24.5 ± 6.8
ABSORBED WITH <u>C. ALBICANS</u> AT 37°C	ABSORBED WITH <u>C. ALBICANS</u> AT 37°C	ADSORBED WITH FRESH MOUSE SERUM AT 4°C	10.7 ± 1.3

¹STANDARD DEVIATION OF THE MEAN OF AT LEAST TWO SEPARATE EXPERIMENTS RUN IN DUPLICATE EACH TIME.

²RABBIT ANTI-C. ALBICANS WAS ABSORBED 3X WITH ZYMOSAN AT 37°C. C. ALBICANS WAS THEN ADSORBED WITH THIS ANTISERUM, WASHED, AND USED IN THE PHAGOCYTOSIS TEST SYSTEM.

³RABBIT ANTI-C. ALBICANS WAS DILUTED IN M199 TO A CONCENTRATION WHICH SLIGHTLY AGGLUTINATED YEAST CELLS. THIS DILUTED ANTISERUM WAS ABSORBED 3X WITH C. ALBICANS AT 4°C. YEAST CELLS WERE THEN SUSPENDED IN THE ABSORBED ANTISERUM TO GIVE A CONCENTRATION OF 4×10^6 CELLS/ML. FRESH MOUSE SERUM WHICH HAD BEEN ABSORBED 3X WITH C. ALBICANS AT 37°C WAS ADDED TO GIVE A CONCENTRATION OF 5% SERUM.

⁴FRESH MOUSE SERUM WAS ABSORBED 3X WITH C. ALBICANS AT 4°C.

⁵FRESH MOUSE SERUM WAS ABSORBED 3X WITH C. ALBICANS AT 4°C. THE ABSORBED SERUM WAS HEAT TREATED AT 56°C, 30 MIN BEFORE USE.

TABLE 8. PHAGOCYTOSIS PROMOTING ACTIVITY OF FRESH MOUSE SERUM AFTER PASSAGE THROUGH AN AFFINITY COLUMN.¹

TREATMENT OF FRESH MOUSE SERUM	% PHAGOCYTOSIS
PASSED THROUGH CONTROL COLUMN	89.7 $\pm$ ³ 6.0
PASSED THROUGH CONTROL COLUMN THEN HEAT-INACTIVATED ²	28.0 $\pm$ 9.9
PASSED THROUGH SEPHAROSE 6B-PE AFFINITY COLUMN	86.2 $\pm$ 9.3
PASSED THROUGH SEPHAROSE 6B-PE AFFINITY COLUMN, THEN HEAT-INACTIVATED	32.6 $\pm$ 10.0

¹1ML OF FRESH NORMAL MOUSE SERUM WAS WASHED THROUGH THE APPROPRIATE COLUMN WITH M199, 10 ML WAS COLLECTED AND USED DIRECTLY IN THE PHAGOCYTOSIS ASSAY.

²THE M199-SERUM COLLECTED FROM BOTH COLUMNS WAS HEATED AT 56°C FOR 30 MIN PRIOR TO USE.

³STANDARD DEVIATION OF THE MEAN OF AT LEAST TWO SEPARATE EXPERIMENTS RUN IN DUPLICATE EACH TIME.

DISCUSSION

Phagocytosis of particulate matter by phagocytic cells involves attachment with subsequent ingestion of the particle by the phagocyte; both steps may be dependent upon serum components. In this thesis some of the conditions required for phagocytosis in vitro were defined and serum factors required for ingestion of Candida albicans by mouse peritoneal macrophages were identified.

Initial experiments were done to determine the optimum incubation time and serum concentration for in vitro phagocytic activity. In some laboratories, workers have used as much as 30% serum in their in vitro test systems (27), whereas we found that a concentration of 2.5% supports optimum phagocytosis. Our data correlate with Leijh, et al. (30), who observed a high rate of ingestion of C. albicans by human monocytes when more than 5% fresh serum was used. Incubation times used by many workers have also varied significantly (27,29,38). Examination in our laboratory of Giemsa-stained cells by light microscopy and gold-coated cells by scanning electron microscopy showed that by 15 min incubation most of the yeast were only attached to the cell membrane of the phagocytic cells. By 1 hr incubation maximum ingestion of C. albicans had occurred. For the remainder of the studies a final serum concentration of 5% was used in the test system and macrophages were incubated in the presence of yeast cells for 1 hr at 37°C.

The use of fresh mouse serum in the test system supported a level of phagocytosis three to four times that supported by serum inactivated at 56°C. Others (27,30) have also observed a decrease in the phagocytosis of C. albicans by human monocytes when heated serum was used. Preheated serum, however, supported greater phagocytic activity than when serum was omitted from the test system; a finding which is in accordance with those reported elsewhere (30). Because of this it became apparent that both heat stable and heat labile phagocytosis promoting factors are present in mouse serum. Furthermore, it was found that serum absorbed with C. albicans did not support the phagocytosis of C. albicans any better than a serum-free system. Therefore, it appeared that the heat-stable and heat-labile serum factors were opsonins in that by adsorbing to C. albicans the yeasts became readily phagocytized. These serum requirements of phagocytosis are not unique to the strain of C. albicans used in these studies since it was found that similar factors are required for the uptake of other strains of C. albicans and for non-candida yeasts and yeast-like fungi.

Complement component C3 and immunoglobulins are opsonins for many different particles (7,10,31,32) and it is known that macrophages bear receptors for the Fc portion of immunoglobulins and the third component of complement (1,13,21,25,31). Our data (Table 3) indicate that C3

is the major serum component which adsorbs to the yeast cells, but IgG and IgA are also detectable. By treating the serum in various ways, attempts were made to identify which of these serum components promotes the phagocytosis of C. albicans.

The opsonic activity of mouse serum was lost by treatment with anti-C3. The anti-C3 serum appeared to be specific for the C3 component of mouse serum because: (1) when tested against whole mouse serum it produced a single precipitin band in Ouchterlony double diffusion plates; (2) the band did not form lines of identity with antisera to other known components of mouse serum; and (3) it produced a single precipitin band coincident with the β_1C fraction of serum in immunoelectrophoresis. These results indicate that the heat labile opsonic factors are related to C3.

It has recently been shown (51,58) that C. albicans activates the alternative pathway of complement - a pathway dependent upon C3 as well as other serum components. To determine if the alternative pathway is important in the phagocytosis of C. albicans, various components of the alternative pathway were removed or inactivated and the treated sera were then used in the phagocytosis assay system. Serum heated at 50°C, a treatment which destroys factor B of the alternative pathway (12), supported an intermediate level of phagocytosis. Similar phagocytic activity was observed if the serum was

pretreated with Zymosan at 17°C which, according to others (47), selectively removes properdin from serum thus inactivating the alternative complement pathway. Serum treated with Zymosan at 37°C, a treatment which reportedly eliminates C3 as well as other early acting components of the alternative pathway (43), was found to be without phagocytosis promoting activity. The importance of the alternative pathway in the phagocytosis of C. albicans was also supported by experiments involving chelators of divalent cations. Both calcium and magnesium cations are required for the activation of the classical pathway of complement activation whereas magnesium but not calcium is required for the alternative pathway. Serum pretreated with a chelator of both calcium and magnesium cations (EDTA) did not support phagocytosis, but serum treated with a chelator of only calcium cations (EGTA) supported optimum activity (Table 4). Appropriate controls indicated that these results were not due to deleterious effects of EDTA on macrophages (Table 5). From these observations we propose that when fresh serum is used Candida albicans activates the alternative pathway of the complement cascade resulting in the adsorption of C3b to the yeast cell wall. The presence of C3b on the cell wall acts as an opsonin probably via macrophage C3b receptors which promotes optimum phagocytosis of C. albicans by mouse peritoneal macrophages. If components required for the activation of the alternative pathway are

removed by various means, C3 will still adsorb to C. albicans (Table 4) and this serum will support an intermediate level of phagocytosis. Because complement activation is prevented, we believe that native C3 is actually adsorbing onto yeast cells and 25-30% of peritoneal macrophages must have receptors for both C3b and unfragmented C3.

Another possible serum factor, C5, does not appear to be responsible for the activities observed in our experiments. Reports on the opsonization of yeast particles by the fifth component of complement (C5) are conflicting. Workers (22,37) have reported that C5 is the phagocytosis promoting serum factor required for uptake of Baker's yeast by phagocytes while others (54) have reported that C5 is not required for the phagocytosis of either Baker's yeast of Candida albicans. Although PMN were used in the latter studies, our data correlate well with those of Rosenfeld et al. (54) since we found that C5-deficient mouse serum supported optimum phagocytosis. Our contention that C3 is important in the uptake of C. albicans also correlates with recent clinical evidence in that uptake of C. albicans by phagocytic cells was found to be low when either human C3-deficient serum (44) or serum deficient in C3b inhibitor (64) was used in the in vitro test system.

For the following reasons we do not believe that IgG specific for C. albicans is responsible for the observed intermediate phagocytic

activity. The phagocytosis promoting activity of serum did not diminish after treatment of the serum with anti-IgG. However, because the serum in these experiments was treated directly with anti-IgG rather than removing the IgG from serum, this evidence by itself does not rule out the possibility that some of the IgG may retain opsonic activity. But, treatment of the serum with anti-C3, which appears to be specific for C3, eliminated the phagocytosis promoting activity of mouse serum. Furthermore, it has been reported that IgG coated particles will bind to the Fc receptor on macrophages in the absence of divalent cations and hence the particles will be ingested (25), but we found that EDTA treated serum was not active. On the other hand, the interaction of a C3b-coated particle with a macrophage is dependent upon magnesium cations (25). Calcium ions are not required for the uptake of C3b-coated particles and in accordance with this it was found that EGTA treated serum was fully active. Treatment of mouse serum with Zymosan depleted the serum of phagocytosis promoting activity, whereas serum containing a known quantity of anti-C. albicans antibody retained the ability to support phagocytosis after Zymosan absorption. The opsonic activity of immune serum was depleted by absorption at 4°C with C. albicans, but mouse serum absorbed at 4°C with C. albicans was still active. An intermediate level of phagocytosis was observed if mouse serum was first absorbed at 4°C, (a

treatment which should remove antibody specific to C. albicans) and then heat-inactivated prior to use. Intermediate activity occurred if yeast used in the test were absorbed with immune serum at 4°C, however, low phagocytic activity occurred if the yeast were preadsorbed with mouse serum at 4°C.

Finally, serum passed through an affinity column to remove antibodies specific for C. albicans and then heat-inactivated, supported an intermediate level of phagocytosis. The functionality of the affinity column is supported by several lines of evidence: 1. The PE used to prepare the column has been shown to be composed primarily of cell wall constituents of C. albicans (6). 2. The agglutination activity (titer) of immune serum against whole cells of C. albicans dropped more than 90% after passage through the affinity column. 3. The immune serum was prepared by immunizing rabbits with whole C. albicans cells, and therefore, the drop in antibody titer indicates that the PE does remove anti-C. albicans antibodies directed against the cell wall components. 4. Elution of antibody from the affinity column with glucose and mannose results in a recovery of 50% of the control antibody agglutinin titer. Therefore, although it appears that C. albicans can be opsonized by the addition of specific C. albicans antibody, our data indicate that antibody is not responsible for any of the activity observed using normal mouse serum.

Our data also show that a serum factor, not absorbable by C. albicans, is required for optimum in vitro phagocytic cell function (Table 5). Thus far we have little information concerning the nature of this third serum component, although we do know that it survives repeated freezing and thawing and temperatures of 60°C (our unpublished data). Others have isolated small peptides from serum which are reportedly responsible for normal in vitro cell functions (42,46). Both peptides, one a tripeptide consisting of the amino acids glycine-histidine-lysine and the other consisting of threonine-lysine-proline-arginine, have been synthesized and are commercially available. We found that neither had an effect on phagocytosis and would not substitute for any of the serum factors that we found to be necessary for phagocytosis of C. albicans (our unpublished observations).

Our findings can be briefly summarized as follows: 1. A serum concentration of 2.5% or greater is required for optimum in vitro phagocytosis of C. albicans; 2. At least three serum components are required for optimum phagocytosis. Two can be absorbed by C. albicans, one of which is heat labile and the other is heat stable. The heat labile component may not be heat labile at all but is C3b, an end product of the heat sensitive alternative pathway of complement activation. The heat stable component is probably C3, 3. Antibody specific for C. albicans can be opsonic, but is not responsible for

phagocytic activity observed in our test system. 4. A third, as yet undefined, factor can not be absorbed by C. albicans, but contributes to the phagocytic process.

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APPENDICES

APPENDIX I

Phagocytosis of C. albicans by congenitally thymic-deficient (nude) mouse macrophages.

The increased resistance of congenitally thymic-deficient (nude) mice to an acute infection with C. albicans has recently been demonstrated (3,9). Nude mice have also been shown to be no more susceptible to experimental mucormycosis than their normal littermates (2). From these studies it has been hypothesized that phagocytic cells may be responsible for this increased resistance, and therefore, an understanding of the opsonins required for the in vitro phagocytosis of C. albicans by nude mouse peritoneal macrophages is needed. Preliminary studies indicate a similarity between the opsonins required for in vitro phagocytosis of C. albicans by normal and nude mouse peritoneal macrophages.

Congenitally thymus-deficient (nude) mice used in this study are from a line crossed into the BALB/c strain. A description of the materials and methods used for studying phagocytosis of C. albicans by nude mouse macrophages is found in the Materials and Methods section of this thesis.

Three levels of phagocytic activity are observed when normal nude mouse serum is treated in various ways (Table 9). These three levels of phagocytic activity coincide with those observed when normal mouse serum is treated in a similar fashion. It is apparent that heat-labile

and heat-stable opsonins are present in nude mouse serum which absorb to C. albicans. Furthermore, no difference in phagocytic activity was observed when either BALB/c macrophages were used with normal or pretreated nude mouse serum or when nude mouse macrophages were used with normal or pretreated BALB/c serum.

Therefore it is concluded that fresh mouse serum is required for optimum phagocytosis of C. albicans by nude mouse macrophages. Our preliminary findings indicate that heat-labile and heat-stable serum factors absorb to C. albicans and render them more readily ingestible by macrophages; a finding identical to that observed when normal mouse serum is used. It therefore seems probable that the opsonins required for the uptake of C. albicans by nude macrophages are similar to those found to be required by normal mouse macrophages for the ingestion of the fungus.

TABLE 9. PHAGOCYTOSIS OF C. ALBICANS USING VARIOUS COMBINATIONS OF SERA AND MACROPHAGES FROM NUDE AND BALB/c MICE.

MACROPHAGE SOURCE	TYPE OF SERUM	% PHAGOCYTOSIS
¹ NUDE	² NUDE	77.0 ± ³ 6.9
NUDE	⁴ HEATED NUDE	10.2 ± 3.3
NUDE	BALB/c	78.5 ± 7.7
NUDE	HEATED BALB/c	16.5 ± 9.1
NUDE	⁵ ABS. NUDE	2.3 ± 1.9
NUDE	NONE	2.8 ± 2.1
BALB/c	NUDE	74.5 ± 7.6
BALB/c	HEATED NUDE	13.1 ± 5.9

¹PERITONEAL MACROPHAGES FROM CONGENITALLY THYMIC-DEFICIENT (NUDE) MICE.

²SERUM OBTAINED FROM NUDE MICE.

³STANDARD DEVIATION OF THE MEAN OF AT LEAST TWO SEPARATE EXPERIMENTS RUN IN DUPLICATE EACH TIME.

⁴NUDE OR BALB/c SERUM WAS HEAT-INACTIVATED AT 56°C FOR 30 MIN, THEN USED IN THE PHAGOCYTIC ASSAY.

⁵FRESH NUDE MOUSE SERUM ABSORBED 3X AT 37°C WITH C. ALBICANS.

APPENDIX II

Effect of PE on the phagocytosis promoting activity of fresh mouse serum.

Phagocytosis of fungi and bacteria can be inhibited by cell components of these organisms. Soluble protein A from S. aureus (6) and staphylococcal cell wall mucopeptides (7) have been demonstrated to inhibit phagocytosis of staphylococci. Dhingra, et al., (4) reported that purified cell wall mucopeptide from S. pneumoniae inhibited phagocytosis of the bacterium, whereas purified pneumococcal capsular polysaccharide did not. Investigators (1,5) have shown that the capsular polysaccharide material from C. neoformans inhibits its ingestion by phagocytic cells. Preliminary studies reported here indicate that a cell wall component from C. albicans inhibits macrophage phagocytosis of this fungus.

A description of the materials and methods used is found in the Materials and Methods section of this thesis. The phagocytic assay, however, was modified in the following way. PE was dissolved in M199 to the desired concentration, the appropriate serum was then added and incubated at 37°C for 10 min. This medium was then used to suspend both the macrophages and yeast cells.

Phagocytosis dependent upon heat-labile and heat-stable serum components was inhibited when PE was added to the test system (Table 10). A much larger concentration of PE was required to inhibit the phagocyto-

sis promoting activity of fresh mouse serum that heat-inactivated mouse serum.

It is difficult to draw any sound conclusions from these data, but it is shown that PE reduces the phagocytosis promoting activity of fresh mouse serum much like whole C. albicans cells. Because C. albicans has been shown to activate the alternative complement pathway (8,10) and since the PE used is composed primarily of cell wall components of C. albicans, the drop in phagocytic activity may be due to the consumption of C3 via activation of the alternative complement pathway.

TABLE 10. EFFECT OF PE ON THE PHAGOCYTOSIS OF C. ALBICANS

¹ CONCENTRATION OF PE IN SYSTEM (MG/ML)	% PHAGOCYTOSIS	
	FRESH MOUSE SERUM	² HEATED MOUSE SERUM
NONE	85.1 ± ³ 6.7	22.0 ± 10.1
4.0	10.5 ± 6.3	⁴ ND
2.0	36.3 ± 10.1	ND
1.0	44.2 ± 12.5	3.8 ± 4.9
0.1	73.9 ± 7.7	3.0 ± 2.8
0.01	80.6 ± 5.2	6.2 ± 2.9
0.0001	ND	9.8 ± 4.1

¹PE WAS DISSOLVED IN M199 BEFORE SERUM WAS ADDED.

²FRESH MOUSE SERUM WAS HEAT-INACTIVATED AT 56°C FOR 30 MIN PRIOR TO USE.

³STANDARD DEVIATION OF THE MEAN OF AT LEAST TWO SEPARATE EXPERIMENTS RUN IN DUPLICATE EACH TIME.

⁴NOT DONE.

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