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 in Microbiology
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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 CL 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 CL 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 CL 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 CL 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 CL 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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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 improtance 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 $37^{o}C$; 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 dificient 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 atnibody 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 of 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 pruchased 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 cource of C3) with anti-C3 were cut from the agar and soaked in 0.01M phosphate buffered saline (pH 7.2) at $4^{o}C$ 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 naimals 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°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°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 of serum was preabsorbed with

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

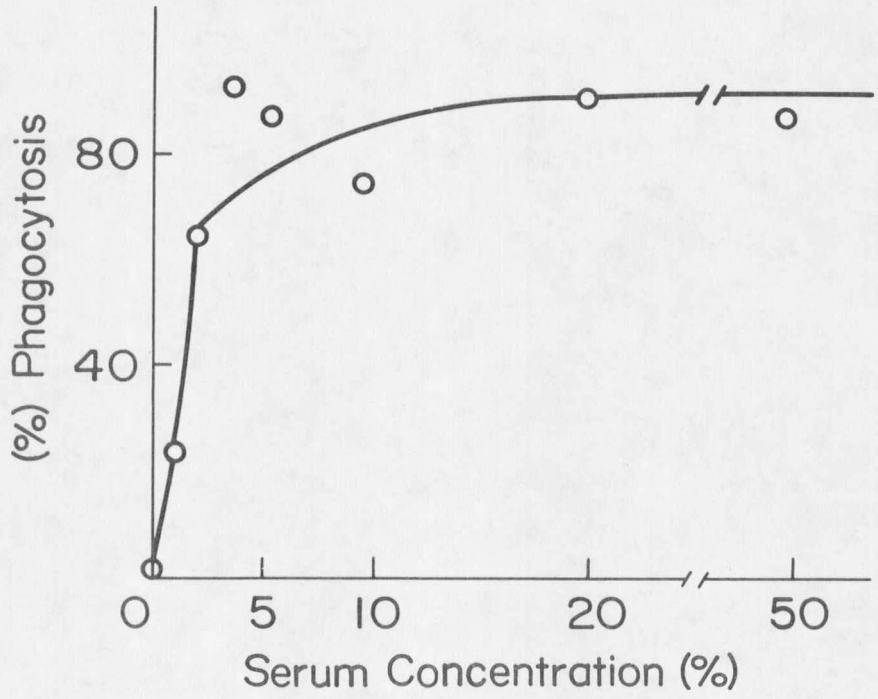
(%) Phagocytosis
80
40
0
5
10
20
50
Serum Concentration (%)

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.